Vol. 36, No. 3 September 1959

The Journal of Experimental Biology

EDITED BY
V. B. WIGGLESWORTH and J. A. RAMSAY

Contents

Published for The Company of Biologists Limited

CAMBRIDGE UNIVERSITY PRESS

BENTLEY HOUSE, 200 EUSTON ROAD, LONDON, N.W.1
AMERICAN BRANCH: 32 EAST 57TH STREET, NEW YORK 22, N.Y.

Price 30s. net (U.S.A. $5.00). Subscription per volume 84s. (U.S.A. $14.00)

ELECTROENCEPHALOGRAPHIC PATTERNS OF THE GOLDFISH (*CARASSIUS AURATUS* L.)*

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(*Received* 5 *August* 1958)

INTRODUCTION

The first recordings of electrical potentials from the teleost brain were made by Adrian and Buytendijk (1931), who used isolated brain stem preparations from *Carassius auratus* L. Slow potential changes (1–3 cyc./sec.) were recorded from the medulla oblongata, corresponding to the rhythm of the goldfish respiratory activity. Patterns of higher frequency and low amplitude were also recorded from the optic lobes.

Enger (1957) has used the codfish (*Gadus callarias*) for implanted-electrode recordings, which were somewhat obscured by artifacts due to breathing movements and to the pressure of the electrodes on the brain surface. He obtained, however, records of the spontaneous electrical activity in the brain of an unanaesthetized fish, as well as mesencephalic responses to stimulation by light flashes. Waves of a frequency of 8–13 cyc./sec. dominated the electrical pattern of the midbrain; these were compared by Enger to the mammalian alpha rhythm. He found evidence for an arousal reaction in the mesencephalon following sudden photic stimulation, but none following auditory stimulation.

In a series of studies of the catfish (*Ameiurus nebulosus*) mesencephalon, Buser (1949*a, b*, 1950, 1951, 1955), Buser & Dussardier (1953) and Buser & Scherrer (1950) analysed tectal responses to electrical stimuli applied to the cut end of the optic nerve; some initial trials were also made using photic stimulation of the intact eye. A complex response to electrical stimulation was recorded from the surface of the optic tectum with monopolar electrodes; it consisted of two groups of components: (1) an initial rapid complex, consisting of one or several rapid diphasic oscillations (duration 2 msec.); followed by (2) a slower component, consisting of one or two larger, slower waves, 20–25 msec. in duration. Upon photic stimulation the first component lasted about 50 msec., the second 100–150 msec. The two components were dissociable; for example, nembutal applied to the surface of the optic tectum abolished the slow response without affecting the rapid component; the slow response was found only on the optic tectum, never along the optic nerve. Buser therefore concluded that this larger, slower component is postsynaptic in origin.

* This investigation was supported in part by Research grants from the National Science Foundation, no. C 4029 (J. P. S.) and no. 37085 (I. J. W.), and The Netherlands Organization for Pure Research (Z. W. O.), no. 96-44-66 (J. P. S.).

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In the present study, the electrical activity of the major brain divisions of the goldfish (*Carassius auratus* L.) was investigated. Effects of anaesthetic, responses to optic stimulation, and pathways of optic impulses within the brain were studied, in the following groups of experiments:

(1) Patterns of spontaneous electrical activity were recorded from various parts of the brain, in curarized and unanaesthetized preparations. Surface recordings were made from telencephalon, mesencephalon, cerebellum and medulla oblongata (Fig. 1). The effect of the level of anaesthesia upon these patterns was studied, using different concentrations of urethane (ethyl carbamate).

Fig. 1. Dorsal view of exposed goldfish brain (camera lucida drawing). Horizontal line indicates 0·5 cm. Bipolar leads were made between: 1, left and right telencephalon; 2, two points of left or right telencephalon; 3, left and right mesencephalon; 4, various points of left and right mesencephalon; 5, two points of cerebellum; 6, left and right medulla oblongata; 7, two points of left or right medulla.

(2) Repetitive light flashes were used to elicit an arousal reaction.

(3) The eye was stimulated by single light flashes and responses were recorded in the four parts of the brain listed above. The optic nerve was then cut on one side, and spontaneous electrical activity and responses to single photic stimuli were subsequently recorded from the same brain divisions.

(4) Spontaneous electrical activity and responses to single light flashes were recorded locally from small areas on the surface of the optic tectum. Following this, one-quarter to three-quarters of the retina was destroyed by electrocautery, and responses were again recorded from the same tectal areas, in an attempt to describe the organization of retinal projection on the midbrain.

(5) Microelectrodes were used to record the response to photic stimulation from deeper layers of the optic rectum.

MATERIALS AND METHODS

Operations

Seventy goldfish, averaging 13 cm. in length, were used in these experiments. Each fish was wrapped in wet gauze and held firmly in a screw clamp just behind the gills, with the lower jaw resting in a curved plastic trough. The animal was usually

anaesthetized with urethane (0·5–8% in different experiments); the anaesthetic solution was continuously dripped from an elevated vessel, through a tube, into the mouth and over the gills. When potentials were to be recorded from an unanaesthetized subject, the fish were immobilized by intramuscular injection of D-tubocurarine (Squibb's 'Intocostrin'), using 0·4 mg./100 g. body weight.

To expose the brain for recording, the skin was peeled away and the bony brain case was gently removed in small pieces, with a pair of jewellers' forceps. Operations were performed under a binocular dissecting microscope with adjustable magnification.

Recordings

Recordings were made with an Offner electroencephalograph; for surface recordings, silver-silver chloride wires were used initially; later, fine-pointed cotton wicks moistened with physiological saline were employed for more precise localization. Microelectrodes for deep recordings were made from tungsten wire 125 μ in diameter, electrolytically sharpened. A modification of the method of Hubel (1957) was used. The wire was suspended between two hooks; it passed through a hole 5 mm. in diameter in a nickel plate, in which was suspended a drop of 2M-KOH; the nickel plate and one hook were connected with a 6 V. a.c. source. Electrolytic erosion of the wire in the KOH solution resulted in a pair of electrodes which separated in the drop. Tips electrolytically sharpened in this manner are 1–5 μ in diameter.

The tungsten wires were then soldered to a thin copper wire for connexion with the apparatus. The assembly was coated by immersion in Glyptal 1201 Enamel (General Electric), and baked at 125° C. for 1–2 hr. A second coat was applied if necessary. Insulation was tested by immersing the electrode in a 1% NaCl solution and passing direct current (9 V., electrode negative) through the solution. Hydrogen bubbling was observed only from the uninsulated tip, under the dissecting microscope, if insulation was adequate. The completed electrodes were mounted on a plastic rod and secured to a micromanipulator.

For photic stimulation, a Grass photostimulator (PS2) was employed. Flash duration was 10 μsec., intensity range between 6 and 100×10^4 f.c. Frequency of stimulation could be varied between 1 and 100 flashes per second.

Responses were also recorded with a Dumont dual-beam oscilloscope; the flash of the lamp was synchronized with the start of the sweep of one beam, the other beam was used as a time signal. Recordings of the oscilloscope picture were made with a Dumont kymograph camera on photosensitive paper.

RESULTS

(1) *General characteristics of spontaneous electrical activity of the goldfish brain*

The spontaneous electrical activity of various parts of the goldfish brain was investigated in the following stages of activity:

(*a*) Without anaesthetic: fish immobilized by injection of Intocostrin, or allowed to recover from light anaesthesia by leading fresh water over the gills for 15 min.

In the latter case, spontaneous tail movements were a sign of recovery from narcosis.

(*b*) 'Stage I' anaesthesia: a light stage of anaesthesia, using $\frac{1}{4}$–2% urethane. The fish did not react to touch, but did react to more severe stimuli such as introduction of a hypodermic needle into the abdominal wall.

(*c*) 'Stage II' anaesthesia: a deep stage of anaesthesia, using 3–5% urethane. The fish reacted to neither touch nor injury (such as needle prick).

(*d*) 'Stage III' anaesthesia: very deep anaesthesia; 6–8% urethane. Recordings were made both in a darkened, quiet room and in a lighted room with normal laboratory activity.

Although the patterns obtained varied somewhat in different fish, and in the same fish during the course of a single recording, a general pattern of activity can be described for each part of the brain.

Telencephalon (Fig. 2A, B). The dominant rhythm from the telencephalon of an unanaesthetized fish in a quiet, dark room had a frequency of 4–8 cyc./sec., with an amplitude of 40–70 μV. The patterns of the two halves of the telencephalon were synchronous. A frequency of 9–14 cyc./sec. was also present, becoming relatively more prominent in a lighted room with normal background noise level. Occasionally a very regular, faster rhythm of 35–40 cyc./sec. dominated the whole pattern of activity; it tended to appear during the later part of the recording periods.

Under stage I anaesthesia, the dominant frequency continued to be 4–8 cyc./sec. although faster, lower-amplitude frequencies began to be more noticeable. Under stage II, the pattern slowed to 4–6 cyc./sec., while the amplitude decreased, in general falling from 50 to 20 μV. within a 10 min. period.

Mesencephalon (Fig. 2C, D). A dominant frequency of 7–14 cyc./sec. prevailed on the surface of the optic tectum in a quiet, dark room. The amplitude was higher than in the telencephalon (60–180 μV.). The pattern was synchronized over the two halves of the mesencephalon. This dominant frequency was mixed with higher frequency waves (18–24 cyc./sec.) of lower amplitude. Again, if the room was light and not perfectly quiet, the higher frequency rhythm became more noticeable, although the 7–14 cyc./sec. pattern continued to dominate the picture.

In stage I anaesthesia, the faster frequency component became less noticeable; the 7–14 cyc./sec. rhythm continued to be dominant but its amplitude was lowered. Then, with increasing depth of narcosis, there was an initial decrease in frequency with enhancement of amplitude, followed by a period of constant frequency with decreasing amplitude.

Cerebellum (Fig. 2E). In either light or dark room, regardless of noise level, there was a constant 25–35 cyc./sec. frequency in the cerebellum, of a low amplitude (20–50 μV.). This was not influenced by light anaesthesia. With the oscilloscope, it was also possible to detect an even faster rhythm of 120–180 cyc./sec., with an amplitude of 5–15 μV. Under deeper anaesthesia (stages II, III), the pattern became more irregular, however, with an increasing amount of low-frequency activity.

Medulla oblongata (Fig. 2F). Activity in the vagal lobes of the medulla oblongata was characterized by slow (0·5–2 cyc./sec.) potentials of a low amplitude. Two groups of higher frequency waves were superimposed on this slow rhythm: a pattern of 8–11 cyc./sec., dominant in the dark; and an activity of 20–35 cyc./sec., observed during light narcosis and in a lighted room. This regular, fast activity disappeared under stage II anaesthesia.

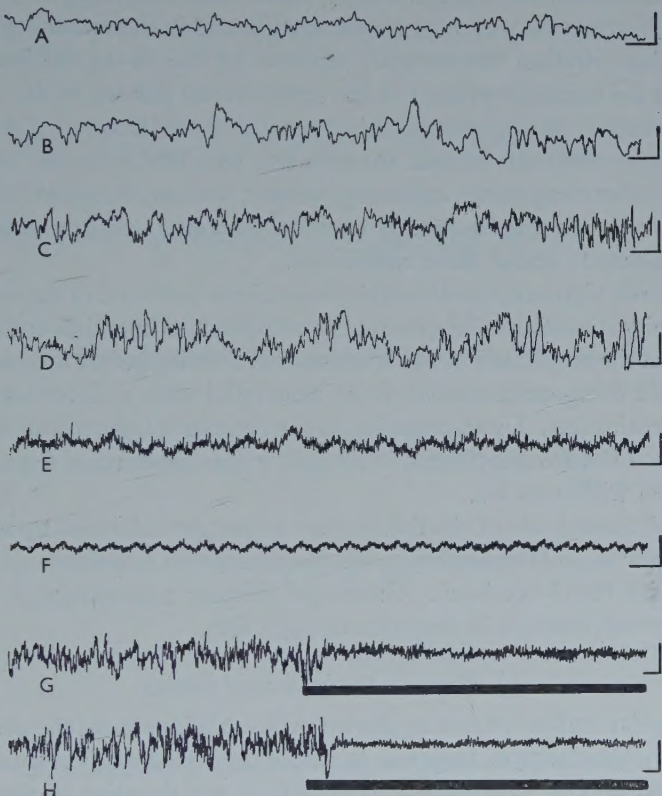

Fig. 2. Recordings from various parts on the brain. A, Recording from two points of right telencephalon; B, recording from left and right telencephalon; C, recording from two points of right mesencephalon; D, recording between left and right mesencephalon; E, recording from cerebellum; F, recording from medulla; G, recording from right mesencephalon; H, recording from left mesencephalon; G and H, horizontal black lines indicate rapid repetitive light flash (75 cyc./sec.) with 20×10^4 f.c. light intensity. Calibration: vertical line indicates 150 μV.; horizontal line indicates 1 sec.

In general, it can be seen from the above results that light anaesthesia does not alter the normal electrical pattern of activity of the teleost brain markedly. For the last three groups of experiments, therefore, a light level of urethane anaesthesia was employed for technical reasons.

It appears that each division of the brain is characterized by a distinct and peculiar pattern of frequencies, probably based upon the cyto-architecture, anatomical connexions, and function of that section of the brain. The lowest frequency was

found in the telencephalon, followed by that in the mesencephalon and then the medulla; the cerebellum had the highest frequency. Amplitude of potentials was highest in the mesencephalon.

(2) *Arousal reaction on photic stimulation* (Fig. 2G, H)

An arousal reaction was obtained in unanaesthetized or lightly anaesthetized fish upon stimulation with repetitive light flashes at a frequency of 50–100 flashes per second. This reaction was most consistently found in the mesencephalon, where the 7–14 cyc./sec. rhythm was abruptly replaced by the 18–24 cyc./sec. frequency. Both rhythms are normally present in the spontaneous pattern of the optic tectum, as previously noted; during arousal, however, the faster frequency dominated the pattern. In some cases this arousal reaction was recorded from the telencephalon; here again, a faster component, normally present, became dominant in response to rapid repetitive photic stimulation. No arousal reactions were found in cerebellum or medulla oblongata under these conditions.

Although some high-amplitude activity sometimes occurred in the first 250 msec. of high-intensity stimulation, in general the amplitude of the high-frequency waves was lower than the amplitude of the spontaneous activity before arousal. This brief high-amplitude component seemed to be associated with a direct response to the high-intensity stimulus. Upon cessation of the repetitive light stimulus, the normal pattern replaced the arousal pattern in all fish, within a period of 1·5 min.; in most fish it occurred within 20 sec.

The level of anaesthesia of the fish had an important influence upon the arousal reaction. Under stage II anaesthesia, the frequency and amplitude of the response was reduced (to 10–18 cyc./sec.). Under the deepest anaesthesia, it was possible to elicit an arousal reaction in only one of eight fish.

(3) *Response to single light flashes*

The monopolar surface recording shown in Fig. 3B illustrates the general characteristics of the optic tectum response to single light flashes. Various areas of the tectum differed slightly with respect to amplitude and duration of the response, as will be shown later.

The latency of the response recorded from the mesencephalon of slightly anaesthetized fish was 30–40 msec. The first part of the response consisted of two to four rapid diphasic spikes, of 40–50 msec. total duration and about 100 μV. amplitude. This complex will be referred to as the 'A complex'. A second portion, the 'B complex', consisted of a large negative spike, with a duration of 90–110 msec. and amplitude 100–200 μV.; it was sometimes preceded by a small positive deflexion, and was followed by a slower positive deflexion of 30–40 msec. duration and 30–40 μV. amplitude. Finally, a slow negative after-potential followed, the 'C complex', with a duration of 50–60 msec. and amplitude of 30–50 μV. The total response lasted 250–325 msec. and was predominantly negative in character.

In the midbrain of a fish in stage II anaesthesia the A complex was not detectable against the background of spontaneous electrical activity; the B complex was

reduced in amplitude. In stage III, the amplitude of the B complex was still further reduced.

With a stimulus of low intensity ($6–15 \times 10^4$ f.c.) no A complex could be detected, and the B complex was of lower amplitude. The effects of low-intensity stimulation were similar in appearance to the response under stage II anaesthesia. At a stimulus intensity of 40×10^4 f.c. or greater the complete response was recorded.

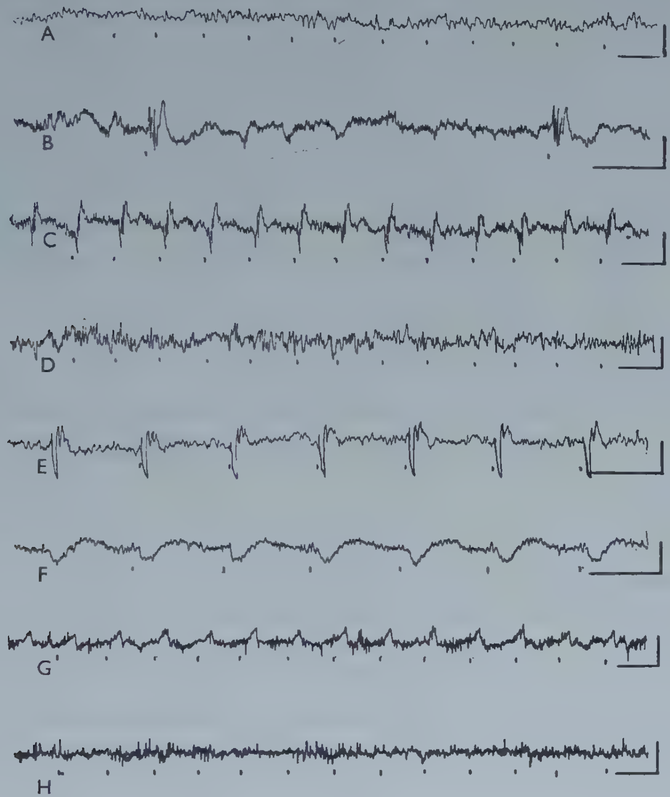

Fig. 3. Recordings from various parts on the brain. A, Bipolar recording from right telencephalon; B, monopolar recording from right mesencephalon; C, bipolar recording from left mesencephalon after cutting left optic nerve; D, bipolar recording from right mesencephalon after cutting left optic nerve; E and F, recording from left mesencephalon, anterolateral portion, before and after cautery of three quarters of the retina, leaving only the superior nasal quadrant intact; G, bipolar recording from medulla. Dots indicate single light flashes, duration 10 μsec., intensity 100×10^4 f.c. Calibration: vertical line indicates 150 μV., except in D where it represents 250 μV.; horizontal line indicates 1 sec.

If the optic nerve of one eye was severed the spontaneous electrical activity of the contralateral lobe was depressed, and no response to light flashes could be elicited (Fig. 3 C, D). Spontaneous activity and responses of the ipsilateral lobe remained unchanged. These findings are in agreement with those of Buser (1949*b*).

No response to a single light flash could be recorded from the telencephalon (Fig. 3A) or medulla oblongata (Fig. 3H). A response was recorded, however, from the cerebellum (Fig. 3G). This was a slow negative wave, with a latency of 60–80 msec., followed by a small positive deflexion. The duration of this response was 60–120 msec.; amplitude was 80–125 μV. (Fig. 6). There was no initial component corresponding to the A complex of the tectum. When one optic nerve was cut, this cerebellar response was usually undetectable; it disappeared also under stage II anaesthesia.

(4) *Localized responses of the optic tectum*

Using fine-pointed cotton-wick electrodes, spontaneous electrical activity and responses to single light flashes were recorded from small areas of the optic tectum. Following this, part of the retina was destroyed by electrocautery and recordings of spontaneous activity and of responses to flashes were made again from the same areas.

Two parts of the mesencephalon departed from the general pattern described above for response to photic stimulation. In the anterior part of the optic tectum the A complex was not detectable against the background activity; the B complex was always present here, however, and its amplitude was higher at the anterior pole than at any other point on the tectum. The lowest amplitude of the B complex—as little as 50% of the amplitude found in other tectal areas—was found at the posterior pole.

Destruction of a part of the retina had the following type of effect on the general photic response pattern (Fig. 3E, F): no A complex could be recorded from some region of the midbrain (in which it had previously been present), and amplitude of the B complex was reduced 40–50% in this region.

Fig. 4 summarizes diagrammatically the results of experiments in which there was destruction of three-quarters of the retina. The left optic tectum is shown, as viewed from above; below it is represented the contralateral (right) eye, with the destroyed portion blackened. On the tectum one circle represents placement of bipolar electrodes in a series of different experiments. Open circles indicate tectal areas where response was unchanged after retinal injury; black circles indicate areas of changed response (40–50% lowering of the amplitude of the B complex, and/or absence of the A complex).

After destruction of all but the superior nasal quadrant of the right retina, the response to single light flashes remained unchanged in part of the posterolateral portion of the contralateral tectum, and at the anterior pole.

After destruction of all except the inferior nasal quadrant, the response was unchanged only in a part of the posteromedial portion of the tectum, and the anterior pole.

After destruction of all except the inferior temporal quadrant, response was unchanged only in part of the anteromedial portion of the tectum.

After destruction of all except the superior temporal quadrant, response was unchanged only in part of the anterolateral portion of the tectum.

In a second group of experiments, only one-quarter of the retina was destroyed, and a search was made for changed response. Results are summarized diagrammatically in Fig. 5, with the left tectum and right eye represented as before.

After destruction of the superior nasal quadrant, the response was changed only in part of the posterolateral portion of the contralateral tectum.

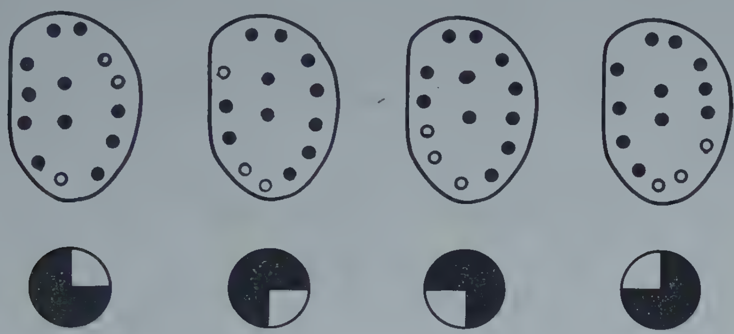

Fig. 4. Schematic representation of results obtained by local bipolar recording before and after cautery of three-quarters of the retina. In the upper row are represented views of the left optic tectum, with circles to indicate electrode placement. Each circle indicates one bipolar electrode placement. Open circles indicate unchanged response; black circles indicate alteration of response. In the lower row, the contralateral (right) eye is shown as viewed from the side of the fish. The blackened segments are those destroyed by cautery.

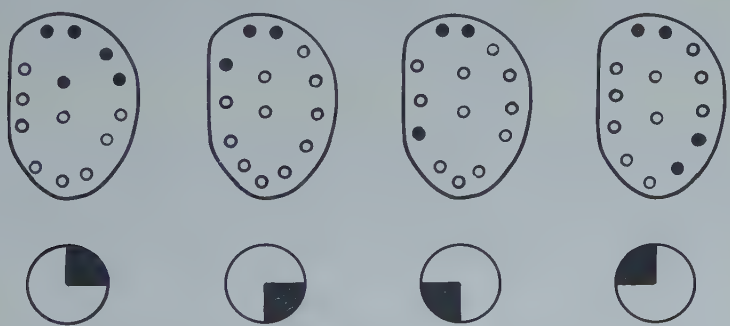

Fig. 5. Schematic representation of results obtained by local bipolar recordings before and after cautery of one-quarter of the retina. Left tectum and right eye as in Fig. 4.

After destruction of the inferior temporal quadrant, the response was changed in part of the anteromedial portion of the tectum, and at the posterior pole.

After destruction of the superior temporal quadrant, the response was changed in part of the anterolateral portion of the tectum, and at the posterior pole.

The results obtained in the two sets of experiments, summarized in Figs. 4 and 5, show localizations of changed or unchanged response which are approximately complementary.

The anterior and posterior portions, and the middle portion of each lobe, were affected in the same way by destruction of any portion of the quadrant, in contrast to the localized changes found at the margins of the tectum. Possible reasons for this will be discussed later.

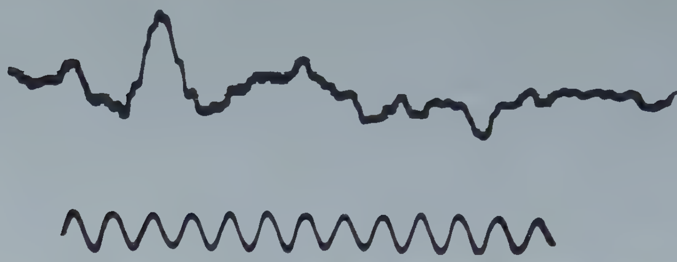

Fig. 6. Bipolar recording from the surface of the left cerebellum. Photograph with Dumont kymograph. (Upwards: negative.) Time signal: 40 cyc./sec., 40 μV. Single light flash at the beginning of the recording.

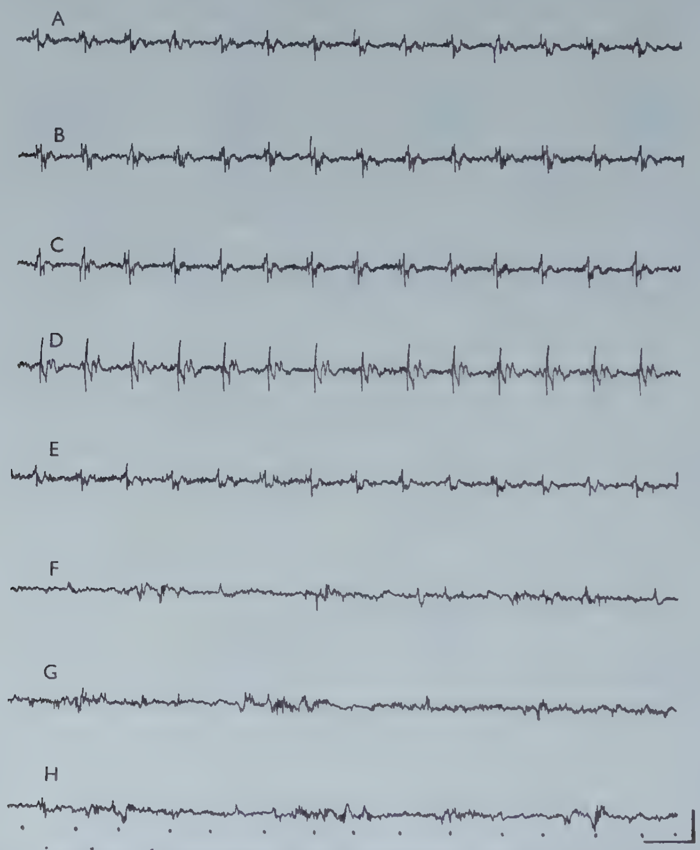

Fig. 7. Bipolar microelectrode recordings from the posterior part of the tectum opticum. A, 40; B, 80; C, 120; D, 160; E, 200; F, 240; G, 280; H, 320 μ under the surface of the tectum opticum. Dots indicate single light flashes, duration 10 μsec., intensity 100×10^4 f.c. (for all records). Calibration: vertical line indicates 150 μV.; horizontal line indicates 1 sec.

(5) *Recordings with microelectrodes*

The response to photic stimulation, as described in general form above, was also obtained from deeper layers of the optic tectum. A maximum (especially of the B complex) response was recorded between 100 and 200 μ, when electrodes

were placed in the middle and posterior part of the tectum (Fig. 7); deeper than this there was no recognizable response. The response was recorded as deep as 2000 μ when electrodes were inserted at the anterior pole of the tectum (Fig. 8); this reflected the fact that vertically inserted microelectrodes at this point passed on a tangent to the ventricle, through the grey matter of the curved anterior margin of the optic tectum.

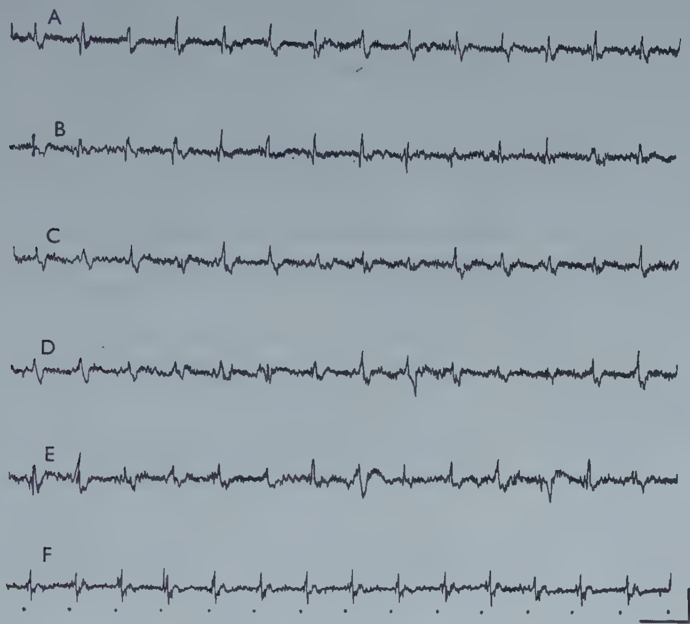

Fig. 8. Bipolar microelectrode recordings from the anterior parts of the tectum opticum. A, 250; B, 500; C, 750; D, 1000; E, 1250; F, 1500 μ under the surface of the tectum opticum. Dots indicate single light flashes, duration 10 μsec., intensity 100×10^4 f.c. (for all records).

DISCUSSION

(1) It was noted in the results that each part of the brain is characterized by a particular pattern of electrical activity. The medulla oblongata was characterized by a slow, low-amplitude rhythm corresponding to the respiratory activity. The highest frequencies (25–35 cyc./sec. and 120–200 cyc./sec. were found in the cerebellum. The telencephalon and mesencephalon were characterized by considerably lower frequencies, of 4–8 and 7–14 cyc./sec., respectively.

The electrical activity of the medulla and cerebellum was not noticeably influenced by light or noise. In the telencephalon and diencephalon, higher frequency waves (9–14 and 18–24 cyc./sec.), which are of low amplitude in a quiet, dark room, were accentuated when the recording room was light and moderately noisy.

Light anaesthesia did not influence the electrical activity of the medulla or cerebellum. Under deep anaesthesia the frequency of the cerebellar activity decreased. In both telencephalon and diencephalon the higher frequency waves became less noticeable under light urethane anaesthesia, and the amplitude of the

dominant lower frequency waves was lowered. As anaesthesia was further deepened there was an initial lowering of frequency with enhancement of amplitude of the dominant waves, followed by a period of constant frequency with decreasing amplitude. This pattern follows the sequence of changes in the cortex of the rat during the progression of urethane narcosis, described by Schadé (1957).

Enger (1957), who recorded a dominant frequency of 8–13 cyc./sec. from the codfish mesencephalon, compared this pattern with the mammalian alpha rhythm. He pointed out that the 8–13 cyc./sec. rhythm recorded in the codfish alternated with spindles, and that it was accentuated in quiet and dark but suppressed in noise and light. It was also more pronounced under light anaesthesia. The mammalian activity shows similar features. He pointed out that the little differentiated paleothalamus of fish may be homologous with the thalamo-reticular system of mammals, and speculated that the presence of the 8–13 cyc./sec. rhythm in the codfish mesencephalon may be associated with the thalamo-reticular system of fish, as the alpha rhythm is believed to be associated with the thalamus of mammals, through a reverberating cortico-thalamic circuit.

The patterns of electrical activity recorded in the two halves of the optic tectum were synchronous. Furthermore, the two halves of the telencephalon produced synchronous patterns. Since the commissural system between the halves of the mesencephalon did not conduct evoked potentials from one half of the mesencephalon to the other, in the experiments on monocular blinding, it seems unlikely that these commissures are involved in the synchronization of the two halves. Another possibility is that the electrical activity of both halves is governed by a more ventral structure in the brain stem, which is either anatomically or functionally unpaired.

(2) Rapid repetitive flashing consistently caused the appearance of a mesencephalic pattern which resembled the typical mammalian arousal pattern (Moruzzi & Magoun, 1949). There was a suppression of slower activity and accentuation of high-frequency low-amplitude activity; this pattern disappeared within 1·5 min. after cessation of stimulation, and was replaced by the normal pattern of activity. Since the fish has in its paleothalamus a structure which is homologous with the thalamo-reticular system of mammals, a similar thalamo-reticular activating system may be responsible for the arousal in fish as well as mammals.

(3) A response to single-flash stimulation was described from the mesencephalon and cerebellum. The cerebellar response, with a latency of 60–80 msec., can be expected from the anatomical connexions between tectum and cerebellum. According to Kappers (1936), Goldstein (1905), Brickner (1929), Charlton (1933), Leghissa (1955) and other authors, there is a well-developed projection system from tectum to cerebellum consisting of both crossed and uncrossed fibres. Although this cerebellar response has not been noted previously in teleosts, it was shown in cats recently by Fadiga, Pupilli & von Berger (1957).

The response from the optic tectum was described in terms of an initial A complex and a later B complex. The A complex, being the first event in the sequence of the evoked potential, might be thought of as an action potential of the

optic nerve. This initial part of the response pattern was not elicited at the anterior pole of the optic tectum, although optic tract fibres are concentrated in that region. Furthermore, the A complex has a relatively long latency (30–40 msec.). Therefore, the first response recorded can hardly be due to the impulses conducted by the optic fibres, but must be considered as a synaptic potential.

Buser (1950), in his study of the complex mesencephalic potentials caused by electrical stimulation of the cut optic nerve, proposed that synchronized activity of the deeper cell layers is responsible for the B complex. In the present study the B complex was found to increase in amplitude, with respect to the A complex, as microelectrodes were pushed to greater depths of the mesencephalon. A maximum of the B complex was recorded at a depth of about 100–200 μ, which roughly corresponds to the deep cell layer.

According to Leghissa (1955) the thickness of the deeper cellular layer of the tectum, composed of motor and horizontal associative neurons, is greatest anteriorly. This is the region of the highest amplitude of the B complex. The B complex has a reduced amplitude posteriorly, where this layer is thinnest. The greater amplitude of the B complex in the anterior region of the tectum may be an expression of the greater thickness of the deeper cell layers in this area.

When the optic nerve of the goldfish was cut on one side, all responses to photic stimulation were abolished in the contralateral tectum, which is explained by the complete decussation of the optic nerves (Kappers, Huber & Crosby, 1936). There is a well-developed commissural system between the deeper cell layers of the two halves of the optic tectum. However, the B complex, which as discussed above probably develops in the deeper layers of the unaffected side, apparently did not spread through the intertectal commissural system to the affected half.

After the optic nerve is cut the spontaneous electrical activity of the contralateral half of the tectum is reduced; in particular, there is a striking reduction in the low-frequency waves. In the normal fish the amplitude of the spontaneous activity is maintained and even increased in the dark. It can be expected that in the dark afferent excitation is reduced. Therefore, the reduction of spontaneous activity following monocular blinding probably is not due simply to cessation of nerve impulses from the optic fibres. It seems possible that the lowered amplitude of activity observed is due to a spontaneous discharge from the injured ends of the nerve fibres. Unsynchronized bombardment from this source might cause a decrease in amplitude of the spontaneous activity of the tectum and suppression of low-frequency activity. Although the pattern somewhat resembles arousal, it probably is not due to activation of the reticulo-thalamic system since the ipsilateral half of the tectum is not affected.

A continuous flow of impulses from severed nerve fibres in fish, like that suggested here, was postulated by Parker (1934, 1936) to explain the following experiment. If a small tail area of the killifish (*Fundulus*) is denervated by a superficial transverse cut, the denervated area will at first darken, then fade after a few days. If a new transverse cut is then made distal to the original cut, a new dark band will appear between this cut and the edge of the tail, within the already denervated area. The

formation of the dark band in each case is interpreted by Parker as being due to a prolonged discharge of the nerve fibres innervating the melanophores. A cold block stops these injury-caused impulses and causes fading of the darkened area.

The cerebellar response to optic stimulation is abolished by monocular blinding. This might be attributed in part to a 50% reduction in the number of impulses arriving at the cerebellum via tecto-cerebellar paths. If considerable facilitation were needed for this response, this might explain the great effect of a 50% reduction in afferent impulses. Furthermore, this response was found only in fresh preparations and was easily abolished by aging or injury. This may also explain why Buser (1949 *a*) failed to find a cerebellar response to electrical stimulation of the cut optic nerve in catfish.

(4) It was shown that the retinal projection on to the contralateral optic tectum could be roughly localized by the use of methods of electrical recording. Talbot & Marshall (1941) have reported a point-to-point projection of the retina on the visual cortex of the cat and monkey, using electrical recording. An indirect method of mapping the superior colliculus, based on reflex motor responses, was used for cats by Apter (1945, 1946), and for fish by Chauchard & Chauchard (1927 *a*, *b*) and Akert (1947, 1949).

Buser & Dussardier (1953), using electrical recording, have shown that each retinal quadrant of certain teleost fishes sends fibres to a different part of the optic lobe. Their findings were confirmed in a recent study by Gaze (1958) who used a microelectrode to explore the surface of the frog mesencephalon. He mapped points of maximal response to a small (15′′) flashing light source. Maximal responses were used for mapping because responses could be evoked from a single point on the optic lobe by stimulation of an area of 5° to 60° radius in the visual field. Despite the overlapping of receptive fields he was able to show a coarse mapping of the retinal projection, such that points on the naso-inferior quadrant of the retina projected to the posteromedial part of the lobe. Points on the temporo-inferior retina projected to the anterior part of the lobe near the midline. Points on the naso-superior retina projected to the posterior part of the lobe near its lateral edge, while those on the temporo-superior retina projected to the anterior part of the lobe near its lateral edge. Recordings made along the midline of one optic lobe corresponded roughly to light stimuli in the central regions of the contralateral visual field.

A direct method of anatomical localization is based on partial destruction of the retina, followed by Marchi staining of degenerated nerve fibres. This method was used by Lubsen (quoted in Ströer, 1940), by Ströer (1940) for *Salmo salar* and *Clupea harengus*, by Akert (1950) for *Salmo irideus*, and by Leghissa (1955) for *Carassius auratus*. By this method a coarse projection of the retinal quadrants on the tectum has been demonstrated. The optic nerve divides into two fascicles. A medial fascicle, derived from the inferior part of the retina, spreads from the anterior pole of the contralateral tectum on to the dorsomedial surface. A lateral fascicle carries fibres from the rest of the retina. Fibres (contained in the lateral fascicle) from the superior part of the retina radiate from the anterior pole to the lateral

surface of the tectum. The nasal retinal afferents terminate on the posterior pole of the tectum, and the temporal afferents on the anterolateral portion of the tectum.

In the recordings of responses to photic stimulation after partial destruction of the retina a localization of responses from the various retinal quadrants, around the margin of the tectum, was observed. This localization agreed with that found by anatomical methods. It was confined to the margins of the tectum, and responses from the anterior, middle, and posterior portions of the tectum were either not altered (anterior and middle) or always altered (posterior) by partial destruction of the retina.

The following working hypothesis of the retinal projection is proposed, based on the anatomical and electrophysiological findings.

Relevant considerations:

(*a*) The optic tract arrives at the medio-anterior pole of the contralateral lobe of the optic tectum, and its fibres can be seen to spread in a fan-like fashion over the tectal surface, on gross inspection.

(*b*) The A complex, as discussed above, is associated with the synaptic connexions made between entering optic nerve fibres and the tectal neurons.

(*c*) The B complex is a sign of subsequent activity in the deeper cell layers.

We assume, for this hypothesis, that soon after reaching the anterior part of the tectum some of the optic nerve fibres, or their branches, make synaptic connexions with central neurons; they continue to form synapses all along the course of their spread across the optic tectum. This is suggested by the finding that both A and B complexes can be recorded everywhere on the tectum except from the anterior pole. The majority of fibres gradually leave the main optic bundles and end at the margin of the tectum on either side of the bundle, establishing the projection described above. Although some degree of overlapping of this projection can be expected, the gross localization achieved by this anatomical arrangement is detected by electrophysiological methods. The tectal area corresponding with a quadrant mapped anatomically is larger than that mapped by electrical methods, which is a measure of the overlap of the projections.

Since no A complex was led off from the anterior pole of the tectum, there are probably few if any synaptic endings of the optic axons in this area. A large B complex is found here, however, which may be due to a spread of activity into the deeper grey matter from adjacent areas. This same spread of activity in the deeper cell layers, via horizontal associative neurons, also explains why the B complex is reduced, but not abolished, in the tectal areas which are affected by partial retinal destruction.

The middle areas of the tectum, according to this hypothesis, will be synaptically connected with afferents from the central areas of the retina, including all four quadrants. This is supported by the results, since both A and B complexes are found in this middle region when any portion of the retina remains intact. The posterior pole of the tectum, which showed a changed response after any retinal destruction, may receive fibres from all areas of the retina.

A quantitative consideration may be used to resolve the apparent contradiction in stating that the ending of fibres from all retinal quadrants is responsible for the presence of not noticeably changed A and B responses in the middle portion of the tectum, while the same anatomical arrangement is also the basis of a change in the evoked response of the posterior pole after destruction of any quadrant of the retina. If one assumes that the connexions between the thick bundles of optic fibres running over the centre of the tectum and the underlying grey matter are plentiful, then the loss of even a considerable percentage of the afferent impulses may cause changes so slight that they cannot be detected by the relatively coarse electrophysiological methods. On the posterior pole the fibre layer has thinned out considerably, however, and the grey matter is least well developed in this area. A reduction of these more sparse connexions may well result in a change in the evoked potential. If considerable spatial summation were involved in the elaboration of the evoked potential, the effect of even a slight reduction in the number of active nerve fibres could have a major effect at low levels of innervation. A similar reasoning was used to explain the absence of a cerebellar response to light flashes in unilaterally blinded fish.

The coarse, overlapping, localized projection of retinal areas provided by this working hypothesis seems to be in agreement with both anatomical and electrical results.

SUMMARY

1. Bipolar surface electrodes were used to record electrical potentials from the brain of the goldfish, *Carassius auratus* L. Characteristic patterns of spontaneous electrical activity were described for telencephalon, mesencephalon, cerebellum and medulla oblongata. Changes in these patterns under deepening urethane narcosis were noted.

2. Rapid repetitive flashing light caused a change in the normal pattern of the mesencephalon which resembled the mammalian arousal. High-frequency (18–24 cyc./sec.) low-amplitude activity replaced the characteristic low-frequency waves (7–14 cyc./sec.); the lower-frequency activity returned 1·5 min. after cessation of stimulation.

3. Responses to single light flashes were described from the mesencephalon and cerebellum. An 'A complex' from the mesencephalon consisted of two to four rapid diphasic spikes, with a latency of 30–40 msec., duration of 40–50 msec., and amplitude of about 100 μV. A 'B complex' consisted of a large negative wave, duration 90–110 msec. and amplitude 100–200 μV., followed by a small positive deflexion, and a slow negative after-potential. The cerebellar response had a latency of 60–80 msec., duration of 100–120 msec., amplitude of 80–125 μV. The nature of these two components was discussed.

Monocular blinding abolished the response to light in the contralateral half of the optic tectum and in the cerebellum. The amplitude of the low-frequency spontaneous activity characteristic of the mesencephalon was reduced in the contralateral tectum.

4. Regional differences in the tectal response to single light flashes were described. Microelectrodes were used to record the photic response from deeper tectal layers.

After destruction of parts of the retina by electrocautery, a disappearance of the A complex and 40–50% reduction of the B complex was observed in restricted tectal areas. The distribution of the areas of changed response was shown to correspond to a coarse overlapping projection of retinal quadrants of the fish eye on to the contralateral optic tectum. A possible basis for the localization of this projection, in agreement with anatomical and electrophysiological findings, was described.

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THE EFFECT OF POSTERIOR LOBE PITUITARY EXTRACTS ON BLOOD PRESSURE IN SEVERAL VERTEBRATE CLASSES

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(*Received* 15 *September* 1958)

(With Plates 5–7)

INTRODUCTION

In 1895 Oliver & Schaefer showed that pithed cats responded to injections of pituitary extracts with a rise of blood pressure. It was later shown (Howell, 1898) that the active substance was contained in the posterior lobe. Paton & Watson (1912) described a fall in blood pressure in decapitated ducks following injection of posterior lobe extracts. Hogben & Schlapp (1924) confirmed these results and recorded the effect of injections of whole posterior lobe extract in amphibians and reptiles.

When the posterior lobe fractions, pitocin and pitressin, became available it was shown that the mammalian pressor response was evoked by pitressin (Kamm, Aldrich, Grote, Rowe & Bugbee, 1928), pitocin having no significant effect, whereas both pitressin and pitocin evoked a fall in the fowl, pitocin being more potent (Gaddum, 1928).

Since Hogben & Schlapp had shown that the predominant effect of whole posterior lobe extract was pressor in amphibians and placental mammals, while in reptiles and birds it was depressor, the observations of Kamm and Gaddum invited investigations of the responses to pitocin and pitressin in different vertebrate classes with the object of deciding whether the results had phylogenetic significance.

Sawyer & Sawyer (1952) found that the amphibians *Bufo marinus* and *Rana catesbiana* exhibited a depressor response to pitocin, while pitressin had no effect in *Bufo* and evoked a rise, fall or diphasic response in *Rana*. Of reptiles, the alligator (Sawyer & Sawyer, 1952) behaved similarly to *Rana* and the turtle (Woodbury & Abreu, 1944) exhibited a depression to pitocin. Only two birds appear to have been investigated. The fowl (Coon, 1939; Strahan & Waring, 1954) always depresses to pitocin and, except in certain cases of low basal blood pressure and when the preparation is tolerant to pitocin, depresses to pitressin also. The pigeon (Waring, Morris & Stephens, 1956) depresses to pitocin and always shows a rise to pitressin. Feakes, Hodgkin, Strahan & Waring (1950) found that in a monotreme (*Ornithorhyncus*) pitocin evoked a fall and pitressin a rise in pressure. The foregoing information permits the generalization that all land vertebrates below the

placental mammals depress to pitocin and different ones, even in the same class, show a variety of response to pitressin. On the other hand, placentals uniformly show a rise to pitressin and no response to pitocin. Feakes *et al.* were so impressed by the pitocin-depressor response of the platypus that they emphasized this monotreme mammal's affinity to reptiles.

The present paper records observations on a variety of species; the aim being to see whether any phylogenetic significance could be attributed to the responses recorded.

MATERIALS AND METHODS

Observations on the vascular responses to mammalian posterior lobe pituitary fractions (pitocin and pitressin) were made on the following species:

Amphibia—toad (*Bufo marinus*), 4 specimens.

Reptilia—tortoise (*Chelodina oblonga*), 5 specimens; lizard (*Trachysaurus rugosus*), 3 specimens.

Aves—penguin (*Eudyptula minor*), 5 specimens; emu (*Dromaius novae-hollandiae*), 2 specimens; cormorant (*Phalocrocorox varius*), 3 specimens.

Mammalia—possum (*Trichosurus vulpecula*), 4 specimens; wallaby (*Setonix brachyurus*), 5 specimens.

(*a*) *Anaesthesia.* Toads were anaesthetized with intraperitoneal injections of Dial Ciba or they were pithed. The most satisfactory anaesthetic for tortoises was a 10% solution of sodium phenobarbitone administered intramuscularly and then intravenously. The lizards were anaesthetized either with ether or with Nembutal injected intraperitoneally. All the birds were anaesthetized with an aqueous solution of sodium phenobarbitone. For the emus a 25% solution was injected intramuscularly, while the penguins and cormorants were injected initially with a 10% solution intramuscularly and, when sufficiently quiet, anaesthetization was completed with intravenous injections. Possums were anaesthetized with intraperitoneal injections of Dial Ciba (120 mg./kg.) and wallabies with either intraperitoneal injections of Dial Ciba (200 mg./kg.) or intramuscular injections of paraldehyde (2 ml./kg.).

(*b*) Blood pressure recordings were made from a cannulated artery connected to a mercury manometer. Before inserting the arterial cannula heparin (1000 i.u./kg.) was injected into the circulation to prevent coagulation. Injections of pitocin and pitressin (Parke Davis) were made via a cannula inserted in a vein. Dilutions of pituitary extracts were made with frog Ringer for experiments on toads, 0·7% NaCl solution for experiments on reptiles and 0·9% NaCl solution for experiments on birds and mammals. Control injections of saline were made in all animals. Owing to the small size of the toads the largest blood vessels had to be cannulated; without totally excluding large areas of the body from circulation satisfactory preparations were obtained using one of the systemic arteries and the femoral vein immediately before its junction with the anterior abdominal vein. The carotid artery and jugular vein were found to be the most suitable for cannulation in the tortoise and lizard. In the birds blood pressure was recorded from the sciatic artery and injections were

made into either the brachial vein (cormorant) or the femoral vein (penguin and emu). The carotid artery and jugular vein were cannulated in possums and wallabies.

RESULTS

Toad. Feakes's (unpublished) investigation of the responses of the toad to posterior lobe pituitary extracts showed a pressor response to both pitocin (5–30 i.u./kg.) and pitressin (10–100 i.u./kg.); her toads were pithed, or anaesthetized with Dial. Further experiments confirm these results. Pithed and anaesthetized animals respond with a prolonged rise to both pitocin and pitressin (Pl. 5, A) and after a series of injections of equal doses of either pitocin or pitressin the preparation becomes less sensitive [i.e. tolerance develops (Pl. 6, A, B)]. Using similar doses, Sawyer & Sawyer (1952) observed depressor responses to both pitocin and pitressin.

Tortoise. Anaesthetized tortoises respond to injections of both pitocin and pitressin with a slow rise in blood pressure (Pl. 5, B). Tolerance develops with serial injections (Pl. 6, C, D). Pressor responses to pitocin and pitressin were obtained by Feakes (unpublished) in one experiment on a tortoise which had been pithed.

Lizard. Feakes *et al.* (1950), using *Trachysaurus rugosus*, anaesthetized with ether, described a depressor response to pitocin and a pressor response to pitressin. I have been unable to confirm this. In my experiments, with animals anaesthetized with ether or Nembutal, both pitocin and pitressin evoked depressor responses (Pl. 5, C). Tolerance develops with serial injections (Pl. 6, E, F).

Penguin. Pitocin evokes a fall in blood pressure and tolerance develops with serial injections. The response to pitressin was predominantly a fall in blood pressure which was sometimes preceded by a small rise. The depressor response to pitressin was obtained at all basal blood pressures between 2 and 12 cm. Hg and serial injections led to a tolerant state. The qualitative responses to pitocin and pitressin are illustrated in Pl. 5, D, and the tolerance which develops to serial injections in Pl. 7, G, H.

Emu. Two anaesthetized emus exhibited depressions to both pitocin and pitressin (Pl. 5, E). The depressor response to pitressin was obtained at base pressures between 3·4 and 10·4 cm. Hg. No information on the development of tolerance with serial injections was obtained in these experiments.

Cormorant. Small doses of both pitocin and pitressin evoke large blood pressure responses in this species. Injection of pitocin produced an initial sharp depression which was sometimes followed by one or two further falls before the base pressure returned to the pre-injection level (Pl. 5, F). The response to pitressin was a depression, which was sometimes followed by a further fall and/or a rise (Pl. 5, F). Partial tolerance developed with serial injections of pitressin (Pl. 7, I). No tolerance to serial injections of pitocin could be demonstrated.

Possum. Preliminary experiments by Feakes were made on animals anaesthetized with Dial and urethane: a pressor response to pitressin was obtained. Further experiments confirmed this response (Pl. 5, G). It was also found that partial

Table 1. *Qualitative responses to pitocin and pitressin, dose levels and anesthetic for representatives of various vertebrate classes*

(Arrows are used to indicate the type of response where traces were not published.)

		Response to pitocin	Dose level (i.u./kg.)	Response to pitressin	Dose level (i.u./kg.)	Anaesthetic
Amphibia	Bullfrog (*Rana catesbiania*), Sawyer & Sawyer, 1952		10·0	↑, ↓, ↓↑	10·0	Nembutal or pithed
	Toad (*Bufo marinus*): (1) Sawyer & Sawyer, 1952		5·0		5·0	Urethane or pithed
	(2) Woolley		2·5–10·0		1·0–10·0	Dial or pithed
Reptilia	Alligator (*Alligator mississippiensis*) Sawyer & Sawyer, 1952		5·0	↑, ↓, ↑↓	5·0	Nembutal
	Tortoise (*Chelodina oblonga*), Woolley		0·05–1·0		0·5–3·0	Sodium phenobarbitone
	Turtle, Woodbury & Abreu, 1944	↓	Not given			Not given
	Lizard (*Trachysaurus rugosus*): (1) Feakes *et al.* 1950	↓	0·5	↑	0·4	Ether
	(2) Woolley		0·02–0·2		0·1–4·0	Ether or nembutal
Aves	Penguin (*Eudyptula minor*), Woolley		0·001–0·5		0·01–0·5	Sodium phenobarbitone
	Emu (*Dromaius novae-hollandiae*), Woolley		0·005–0·025		0·01–0·025	Sodium phenobarbitone
	Cormorant (*Phalocrocorox varius*), Woolley		0·02–0·8		0·03–0·8	Sodium phenobarbitone
	Pigeon, Waring *et al.* 1956	↓	0·2	↑	0·01	Pentobarbitone
	Fowl (*Gallus domesticus*), Strahan & Waring, 1954		0·08		0·3	Sodium phenobarbitone
Mammalia	Platypus (*Ornithorhyncus*), Feakes *et al.* 1950		0·2–0·4		0·2–0·5	Dial and urethane or pithed
	Possum (*Trichosurus vulpecula*), Woolley	—	—		0·006–0·7	Dial
	Wallaby (*Setonix brachyurus*), Woolley	—	—		0·001–3·0	Dial or paraldehyde
	Rat, Landgrebe *et al.* 1946	—	—		0·015	Dial and urethane

tolerance could be induced with serial injections of pitressin (Pl. 7, J). Injections of small doses of pitocin had no effect on blood pressure; with large doses a rise occurred which could be accounted for on the basis of the 4% pitressin contamination of pitocin.

Wallaby. Pitressin evokes a pressor response and partial tolerance is developed with serial injections. The responses to small and large doses of pitocin were the same as those obtained in the possum.

DISCUSSION

Placentals and marsupials are insensitive to pitocin; all other forms investigated, except *Bufo* and *Chelodina*, depress to pitocin. *Chelodina* exhibits a rise to pitocin; the results reported here on *Bufo* (a rise) are opposite to those reported by Sawyer & Sawyer (1952).

The response to pitressin is much more variable. Amphibia exhibit a fall, rise or diphasic response, reptiles a rise or diphasic response, birds a fall except for the pigeon, and all three subclasses of mammals, a rise. When we had results from onl two birds, pigeon and fowl, we were encouraged to examine others to see whether flying and terrestrial species differed consistently; this is not sustained by results from a cormorant, a flying bird.

The observation by Feakes *et al.* (1950) that, like all reptiles investigated up to that time, a monotreme exhibited a depressor response to pitocin encouraged the earlier suggestion that there might be correspondence between the vascular responses to posterior lobe pituitary extracts and phyletic position. Table 1 shows that this proposition can no longer be entertained. Thus within Reptilia different species of Chelonia exhibit opposite responses to pitocin, and within both Reptilia and Aves different species exhibit opposite responses to pitressin.

Whether the responses under consideration are pharmacological artifacts or have physiological significance is not strictly germane to our object. Nevertheless, when doses are converted to dose level/kg. as in Table 1 it is clear that for mammals and birds the excitant dose is such that it could be supplied by endogenous secretion, that in reptiles it probably could not, and for amphibians almost certainly not. So, sensitivity of peripheral vessels to mammalian posterior lobe excitants has increased with the evolution of higher forms.

SUMMARY

1. The blood pressure responses of representatives of various vertebrate classes to pitocin and pitressin have been recorded.

2. No relationships between phyletic position and the type of responses exhibited to pitocin and pitressin were observed.

3. Tolerance is developed to serial doses of pitocin and pitressin in both cold blooded and warm blooded vertebrates.

I am indebted to M. Feakes for permission to mention unpublished results. Salary and some expenses were met by an N.H.M.R.C. grant to Prof. Waring; some expenses were met from a Western Australian University research grant.

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EXPLANATION OF PLATES

Plate 5. Responses to pitocin (O) and pitressin (P)

A	Toad	(2)	0·25 i.u.	O	(5)	0·5 i.u.	P
B	Tortoise	(15)	1·0 i.u.	O	(10)	1·0 i.u.	P
C	Lizard	(6)	0·05 i.u.	O	(4)	0·1 i.u.	P
D	Penguin	(6)	0·001 i.u.	O	(18)	0·2 i.u.	P
E	Emu	(4)	0·25 i.u.	O	(2)	1·0 i.u.	P
F	Cormorant	(19)	0·2 i.u.	O	(26)	1·0 i.u.	P
G	Possum	(2)	0·1 i.u.	P			

Ordinate: pressure in cm. Hg; abscissa: time; A, G, 5 min. intervals; B–F, 30 sec. intervals.

Plate 6. Responses to serial injections of pitocin (O) an pitressin (P) showing tolerance

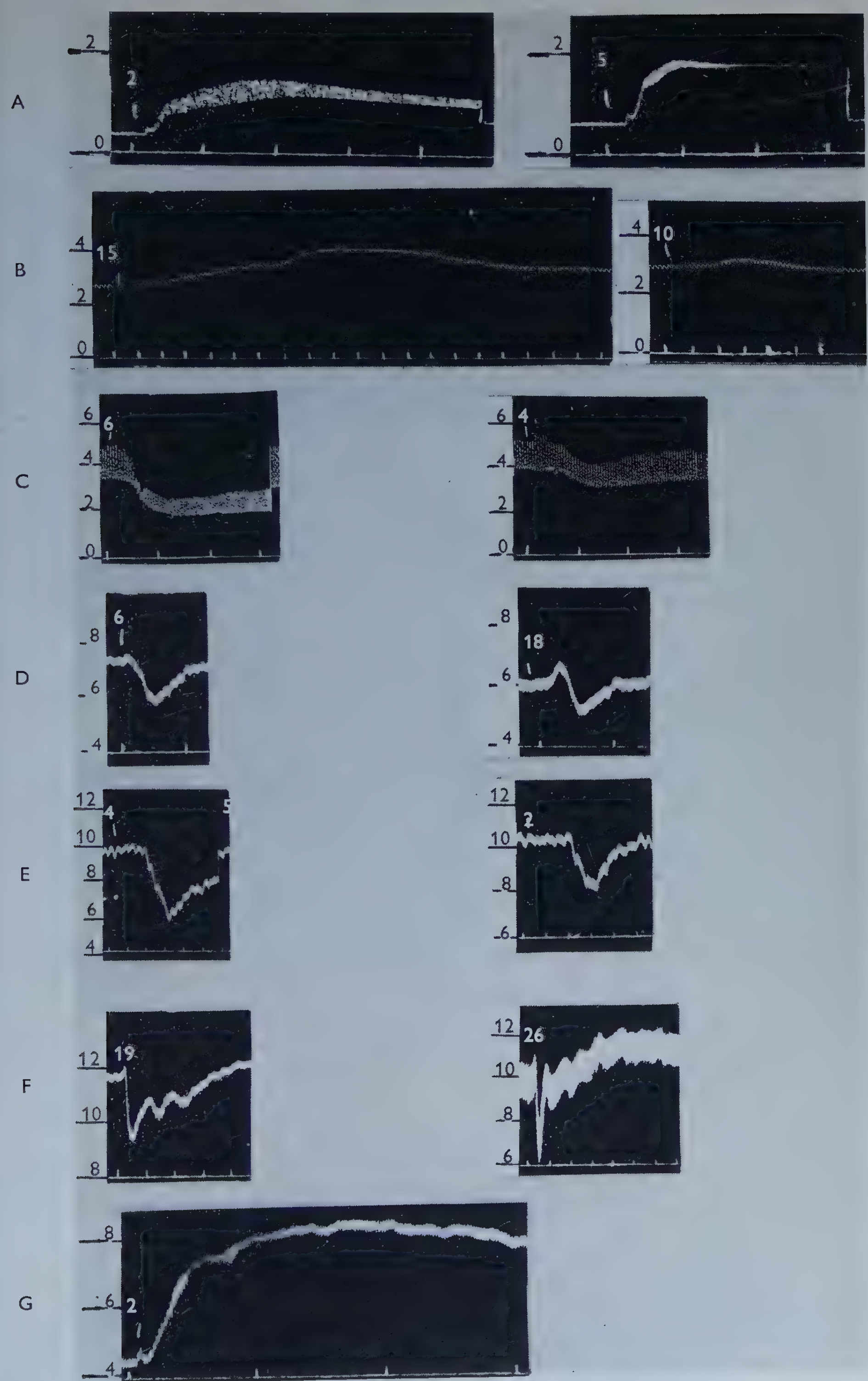
A	Toad	0·25 i.u.	O
B	Toad	0·25 i.u.	P
C	Tortoise	0·5 i.u.	O
D	Tortoise	1·0 i.u.	P
E	Lizard	0·01 i.u.	O
F	Lizard	0·1 i.u.	P

Ordinate: pressure in cm. Hg; abscissa: time; A–D, 5 min. intervals; E, F, 30 sec. intervals.

Plate 7. Responses to serial injections of pitocin (O) and pitressin (P) showing tolerance

G	Penguin	0·1 i.u.	O
H	Penguin	0·2 i.u.	P
I	Cormorant	1·0 i.u.	P
J	Possum	0·2 i.u.	P

Ordinate: pressure in cm. Hg; abscissa: time; G–I, 30 sec. intervals; J, 5 min. intervals.



WOOLLEY—THE EFFECT OF POSTERIOR LOBE PITUITARY EXTRACTS ON BLOOD PRESSURE IN SEVERAL VERTEBRATE CLASSES

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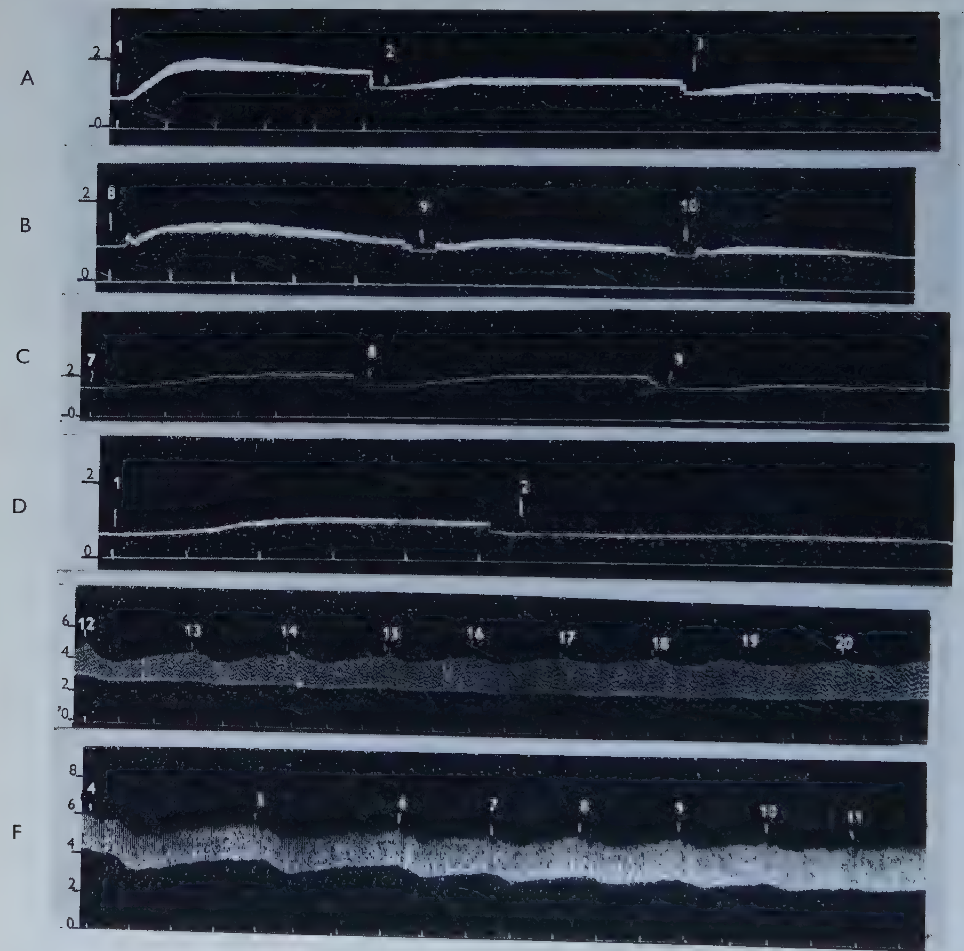

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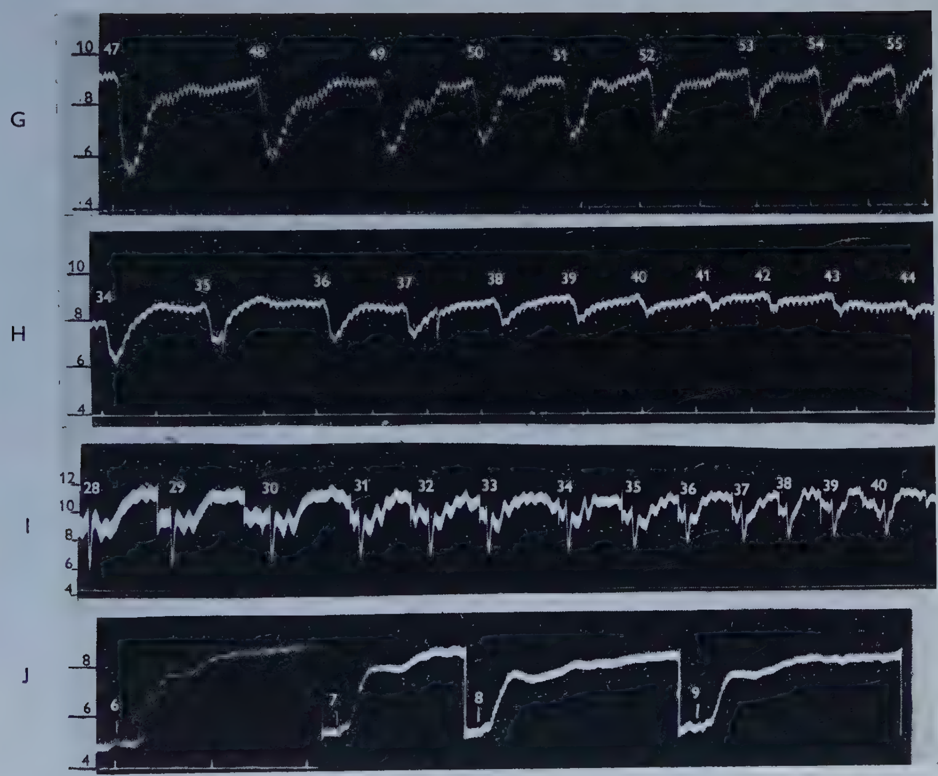

WOOLLEY—THE EFFECT OF POSTERIOR LOBE PITUITARY EXTRACTS ON BLOOD PRESSURE IN SEVERAL VERTEBRATE CLASSES

THE CUTICULAR PATTERN IN AN INSECT, *RHODNIUS PROLIXUS* STÅL

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(*Received* 17 *February* 1959)

(With Plates 8–10)

INTRODUCTION

In the larval stages of *Rhodnius* the abdominal cuticle between the intersegmental membranes is covered with a pattern of stellate pleats among which are set the bristle-bearing plaques (Pl. 8, fig. 1). This stellate pattern results when the newly formed cuticulin layer is 'thrown into folds'. Wigglesworth (1933) says: 'The folding must be a spontaneous change occurring in the membrane itself, for the folds bear no relation to the arrangement of the underlying cells. The secretion of the cells evidently coagulates at first to form a smooth membrane, which then expands (perhaps by the inbibition of water from the moulting fluid outside it) and becomes folded, falling naturally into stellate folds like those in the skin which appears at the surface of hot milk.' In adult *Rhodnius* the plaques are lost upon the tergites and the stellate pattern is replaced by transverse ridges (Wigglesworth, 1940). The ridges or ripples resemble stellate pleats which have been stretched transversely (Pl. 8, fig. 2) and merge at the margins into the more randomly pleated pattern (Pl. 8, fig. 3). In tracheae the form of the cuticle has also been ascribed to the expansion and buckling of the first formed cuticulin layer (Wigglesworth, 1954*a*; Locke, 1957, 1958*a*). A very simple model shows how expansion and buckling can cause a stellate pattern. The epithelium was represented by a flat sheet of 25% gelatin and the cuticulin layer by a thin film of rubber latex. The latex was then caused to swell by immersion in xylene. The expanding rubber took a form resembling the larval stellate pattern, distorting the gelatin beneath it (Pl. 8, fig. 4). It seemed probable that the ripples of the adult cuticle could arise similarly with the addition of an axial or transverse orienting stress. Rubber and gelatin models were made to swell after the block had been slightly stretched or compressed. This distortion caused ripples not unlike the cuticle in adult *Rhodnius* (Pl. 8, fig. 5). Tension resulted in the elongation of the pleats in the direction of the force and compression caused the formation of ripples at right angles. Thus the adult pattern could result from that in the larva with the addition of axial compression or lateral tension. The experiments to be described resulted from attempts to verify this naïve hypothesis.

METHODS

All experiments were performed upon larvae of *Rhodnius prolixus* Stål. Recently moulted 5th-instar larvae are remarkably resistant to rough surgical treatment and no special antiseptic precautions were necessary. The operations were carried out under a binocular microscope with a mounted fragment of razor blade. With practice, squares down to 100 μ across could be cut and manipulated with a needle in a drop of blood on the tip of the blade. No wax was used to seal the wounds, which hardened with congealed blood in a few hours. The dark rim of blood along the line of cuts was useful in assessing the extent of wounding which when severe might cause anomalous results. Histological preparations showed that after a week at room temperature (25–32° C.) the cells had completed their repairs. The larvae were then fed to initiate moulting and metamorphosis. After most operations it was found necessary to give the larvae two small blood meals separated by several days, rather than the customary single meal causing severe distension and the risk of hernia. The results were observed on the adult cuticle mounted unstained side by side with the exuvium, which provided a perfect record of the operation performed. Pl. 8, fig. 6 shows the exuvium from a typical operation. Between six and twelve larvae were used for each experiment, but conclusions were frequently verified by repeating the operation in a slightly different way. In experiments in which the adult pattern showed no change, the site of the operation could be made out by the remains of tracheae trapped between the host cuticle and the transplant. The tracheal lining resisted digestion by the epithelium and haemocytes and caused small tubercles to outline the cut edges (Pl. 8, fig. 9). Transplanted cuticle could also be distinguished by its different level, since it seldom happened that the graft could be made to fit the hole exactly. Some experiments were performed in earlier instars than the 5th. In transplants there was no indication of incompatibility due to the origin of graft and host from different animals.

RESULTS

The regeneration of the ripple pattern

A simple physical explanation of the adult cuticle pattern is favoured by the results of some wounding experiments. If the ripple pattern is determined by the disposition of individual cells each with a fixed potency, it might be expected that the cell migration which takes place into a wounded region should cause a distortion of the pattern. Wigglesworth (1937, 1940), found pigment patterns disturbed in this way. If, on the other hand, the pattern is caused by the operation of some simple physical force, like expansion and buckling operating over a large area, then migration of cells might have little effect upon the later pattern.

The centre of a tergite of one side was burned by a small drop of stearic acid (melting-point 69° C.). The drop was kept molten for 60 sec. with a hot wire and flattened out to a disk about 0·05 cm. in diameter, well within the intersegmental membranes. The larvae were left for a week for the wound to heal before being fed.

The pattern in the adult showed no distortion, although the burned region could be recognized by its shininess (the dermal glands secreting cement had probably been destroyed) and a slightly greater separation of the ripples.

To confirm this result a square of cuticle was excised from the centre of the tergite of one side and the wound covered with a glass plate to promote healing. The larvae were fed a week later when the cells could be seen covering the glass. The pattern in the adults showed no centripetal displacement.

A different result was observed when the burn or excision was large enough to affect the boundary region of the segment or the intersegmental membrane itself. The cuticular patterns from these regions were displaced towards the centre of the disturbance, showing that migration of cells had taken place (Wigglesworth, 1940).

Thus it seemed that the ripple pattern was not the result of the disposition of cells each with the potentiality for forming only a particular part of a ripple.

Possible factors in ripple orientation

These results suggested that the ripple pattern might be caused by expansion opposed by an axial restraint over a large area, perhaps over the whole of each segment. Many structures, for example the evanescent segmental muscles described by Wigglesworth (1956), could cause such coarse axial restraints.

Text-fig. 1. The effect upon the adult pattern of rotating a square of integument in the larva. The position of the heart in the mid-line is shown by stippling.

To test this a square was cut from the tergite of one side, rotated through 90°, and replaced (Text-fig. 1). The wound healed and the larvae were fed a week later. Now, if the ripple pattern is the result of some simple physical force operating upon the epithelium as a whole, the adult pattern should be unchanged except for the results of injury in the operation. If the adult pattern is determined in the larva at the cellular level the square of cuticle should show traces of the original orientation. Pl. 8, fig. 7 shows that in the adults the ripples on the rotated cuticle remained approximately at right angles to the remainder of the pattern. Thus the orientation of the pattern is determined at the cellular level.

Cuticle symmetry and epithelial asymmetry

The adult ripple pattern has two axes of symmetry, anterior-posterior and side to side. The shape of the ripples in the centre of a segment does not allow differentia-

tion between the anterior and posterior directions, or left and right. A force acting along either axis could produce the pattern—axial compression or lateral tension. The simplicity of the cuticular pattern suggested that there might be an equal simplicity in the cellular mechanism responsible for its orientation.

A small square was cut from the centre of a tergite on one side, rotated through 180°, and replaced. The ripple pattern on such a square should be parallel to that of the surrounding cuticle. If the pattern is oriented by an axial or transverse force or even by both together no discontinuity would be expected at the edges of the square. The result obtained is shown in Pl. 8, fig. 8 and diagrammatically in Text-fig. 2. The ripples in the surrounding cuticle make no contact with the rotated square but are deflected to unite with one another anteriorly and posteriorly. The ripples in the centre of the square retain their orientation parallel to the surrounding cuticle, but those on the periphery take a circular course linking up with themselves. This discontinuity of the pattern at the edges of the square suggested that the epithelium underlying the pattern is not symmetrical about the two axes. This does not preclude the possibility that the ripples in the cuticle may be formed by some simple physical force acting in one direction, but there is no similar simplicity in the epithelium.

Text-fig. 2. The effect upon the adult pattern of rotating a square of larval integument through 180° (cf. Pl. 8, fig. 8; Pl. 10, figs. 19, 22).

The causes of asymmetry

The asymmetry could be ascribed to a polarity or a gradient. The simplest description would suppose that the epithelium is polarized, with a directional property in one or both axes. A more complex theory would suppose that one or two gradients existed. These would have polarity but would differ quantitatively along the gradient. Thus there are several possible descriptions of the asymmetry:

(1) A polarity in the axis;

(2) a transverse polarity;

(3) a compound axial polarity being reversed or interrupted at certain points, for example, the intersegmental membranes;

(4) a compound transverse polarity split, for example, at the sides, mid-dorsally or mid-ventrally;

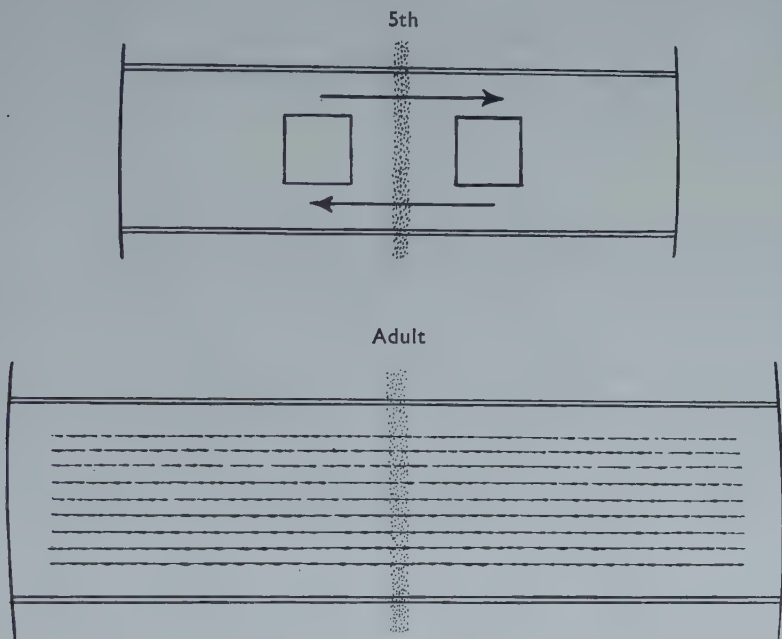
(5) an axial gradient;

(6) a compound axial gradient perhaps varying segmentally;

(7) a transverse gradient;

(8) a compound transverse gradient perhaps varying from the sides, mid-dorsally and mid-ventrally.

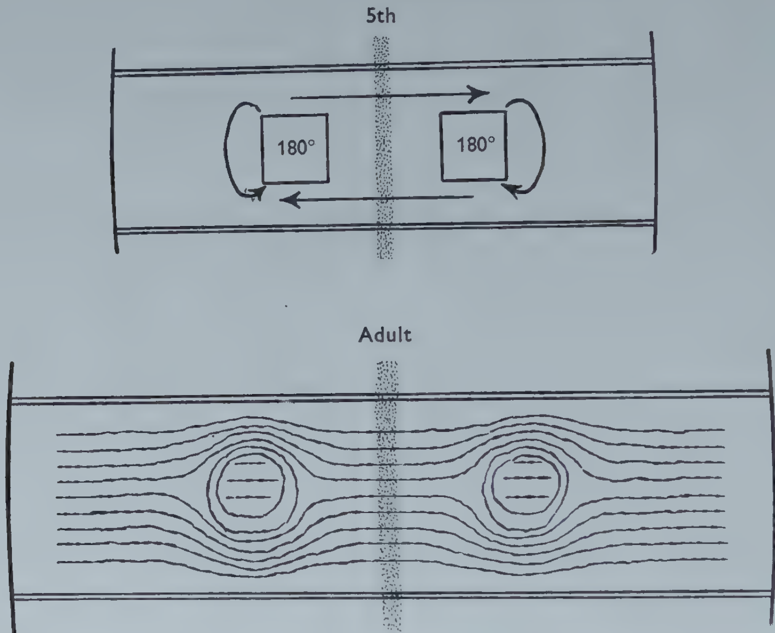
This being so, in a square rotated through 180° four features will have been altered. (*a*) The relative position of all parts except the dead centre of the square will have been altered in the transverse direction with respect to the rest of the animal, and (*b*) in the anterior-posterior direction. (*c*) The orientation will have been reversed in the transverse direction, and (*d*) in the anterior-posterior direction. One or all of these features might be responsible for the discontinuity.



Text-fig. 3. The effect of interchanging squares of integument in symmetrical positions upon either side of the mid-line. The adult pattern is undisturbed (cf. Pl. 8, fig. 9).

Transverse mechanisms. Two squares were cut from the same tergite in symmetrical positions on each side of the mid-line (Text-fig. 3). They were removed and replaced in opposite holes. A handle of soft wax fixed to each square facilitated this operation. In this way two squares were obtained in which the mesial-lateral direction alone was reversed. The pattern in the adult remained unblemished (Pl. 8, fig. 9). Thus reversal of the mesial-lateral direction does not induce the discontinuity seen in squares rotated through 180°. There is, therefore, no evidence for a compound transverse polarity reversing in the mid-line (alternative (4) above). This experiment also negates the possibility of any transverse gradient (alternatives (7) and (8)), for the relative positions have been altered in the transverse direction without inducing discontinuity. Thus all mechanisms operating transversely have been eliminated except a simple transverse polarity.

This result has been confirmed in other ways. Two squares were cut from the same tergite in symmetrical positions on either side of the mid-line as before. The squares were exchanged and rotated through 180° (Text-fig. 4). In this way two squares were obtained reversed except for the mesial-lateral direction. Pl. 9, fig. 10 shows the typical discontinuity pattern produced in the adult.



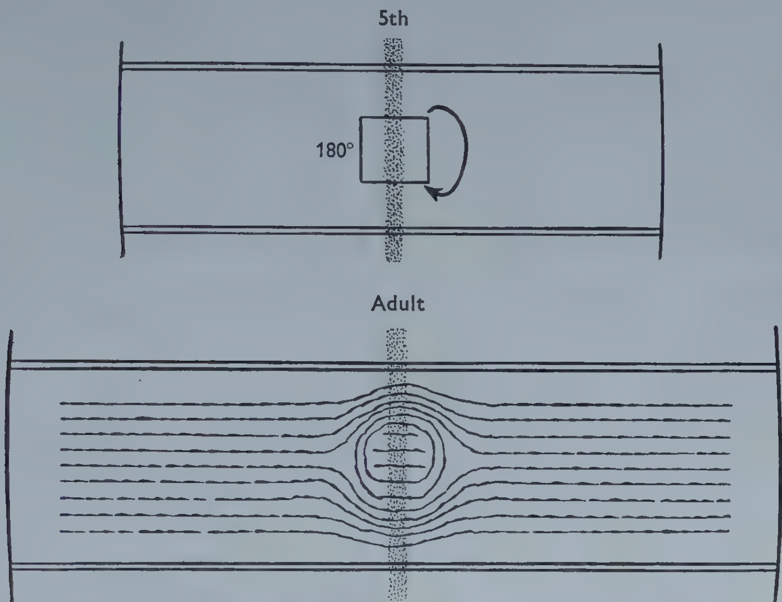
Text-fig. 4. The effect upon the adult cuticle of reversing the anterior-posterior direction in the larva while leaving the mesial-lateral direction unchanged (cf. Pl. 9, fig. 10).

The importance of changes in the axis as causes of the discontinuity pattern has also been confirmed by operations in the mid-line. A square of cuticle was cut in the centre of a tergite in the mid-line. It was removed, rotated through 180°, and replaced (Text-fig. 5). Thus the direction and relative position were altered only in the axis and with respect to a simple transverse polarity and a simple transverse gradient. The typical discontinuity pattern resulted in the adult (Pl. 9, fig. 11).

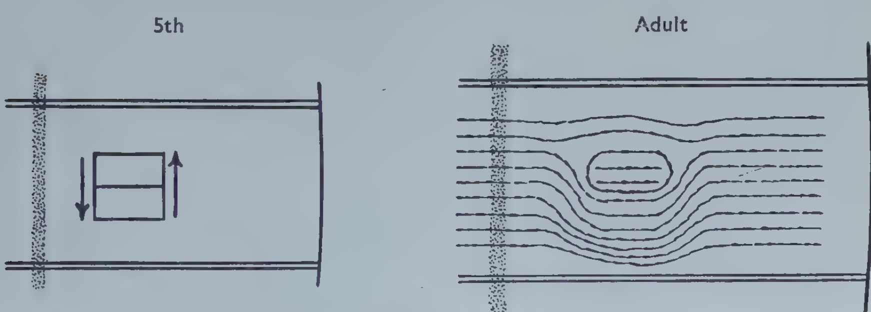
From these experiments it was thought that disturbances in the axis interfered with the normal orientation of the ripple pattern although a simple transverse polarity had not been excluded.

Mechanisms in the axis. If the asymmetry is due to any form of axial polarity then it should not be possible to induce the discontinuity pattern by changes in relative position in the axis. Two rectangles were cut from the centre of a tergite upon one side as in Text-fig. 6. The position of the rectangles was interchanged without altering their orientation. The result was somewhat variable. The most usual result is shown in Pl. 9, fig. 12. The ripple pattern is deflected to unite with the rectangle from the anterior margin of the segment. The other shows the discontinuity pattern. In some preparations both rectangles showed discontinuity

patterns. In either event the asymmetry is not the result of axial polarity (alternative (1) above). Also the discontinuity has been induced without any change in transverse orientation and a transverse polarity cannot be concerned (alternative (2)). The asymmetry is therefore due to axial displacement alone, either in a simple axial gradient or an axial gradient varying segmentally.



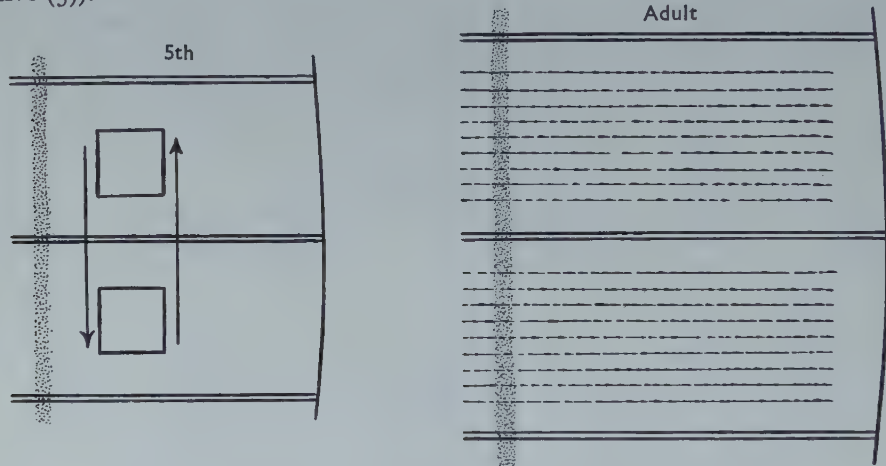
Text-fig. 5. The effect upon the adult cuticle of reversing the anterior-posterior direction in the mid-line where mesial-lateral effects should be symmetrical (cf. Pl. 9, fig. 11).



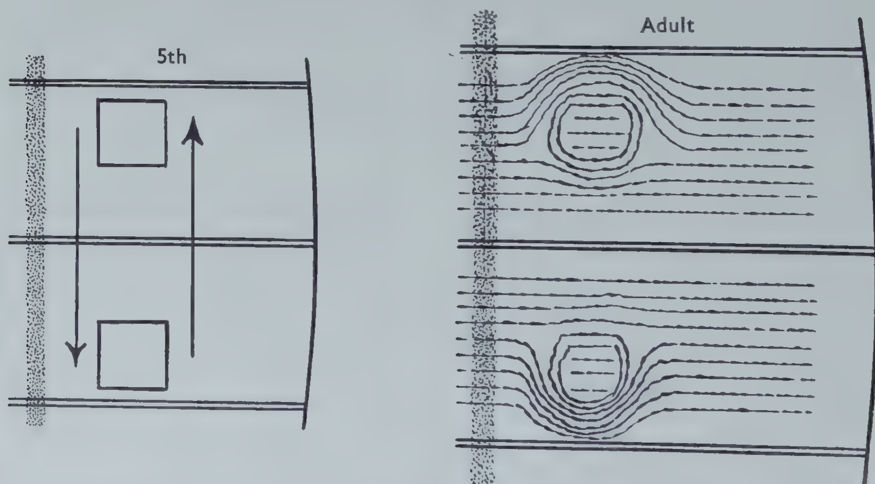
Text-fig. 6. The effect upon the adult cuticle of interchanging rectangles of integument in the axis (cf. Pl. 8, fig. 6; Pl. 9, fig. 12).

If the gradient varies quantitatively in the length of the animal a disturbance of the pattern might be expected in grafts exchanged between segments. If, however, there is a serial repetition of the gradient in each segment, no disturbance might result. A square was cut from the centre of the 4th and the centre of the 5th tergite of one side. The squares were interchanged without altering their orientation (Text-fig. 7). The pattern in the adult showed no trace of discontinuity. This was

not because the epithelium from different segments was incapable of stimulating the discontinuity pattern. When the experiment was repeated, cutting squares towards the anterior and posterior margins of their respective segments as in Text-fig. 8, discontinuity patterns resulted (Pl. 9, figs. 13, 14). Thus the gradient is within each segment (alternative (6)) and not in the length of the animal (alternative (5)).



Text-fig. 7. The effect upon the adult cuticle of interchanging squares of integument from similar positions in adjacent segments.

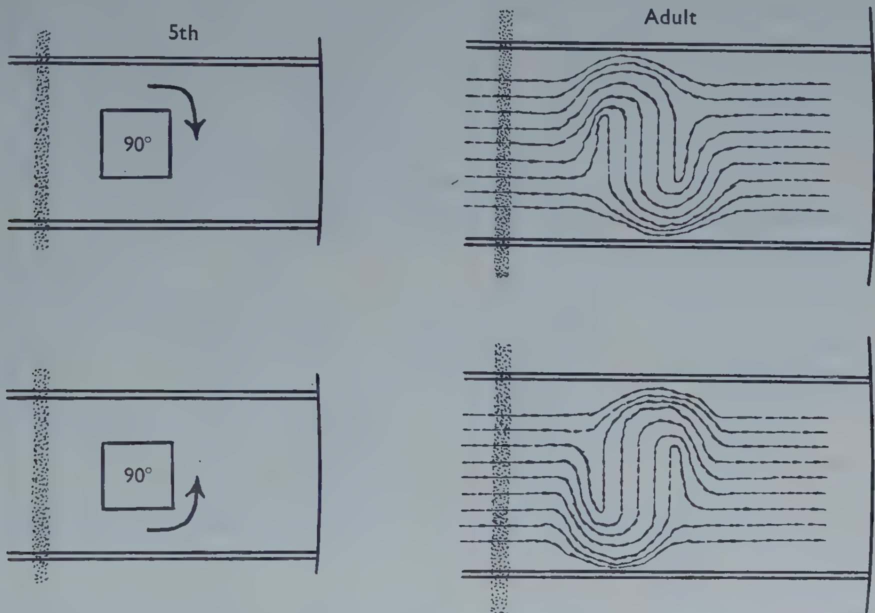


Text-fig. 8. The effect upon the adult cuticle of interchanging squares of integument from adjacent segments when one square is close to the posterior intersegmental membrane and the other is close to the anterior intersegmental membrane (cf. Pl. 9, figs. 13, 14).

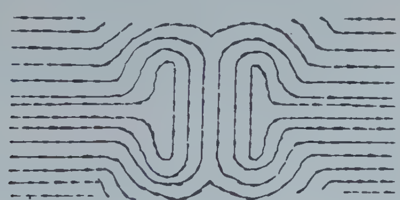
The 'handedness' of the orienting mechanism

The preceding experiments show that within the area of a tergite occupied by the transverse ripples a mechanism of orientation exists in the axis. Within this area cells cannot be interchanged in the axis without affecting the pattern. The

description of this as an axial gradient introduces the possibility of a 'handedness', a distinction of right and left at each of the transverse levels. This possibility is explored below.



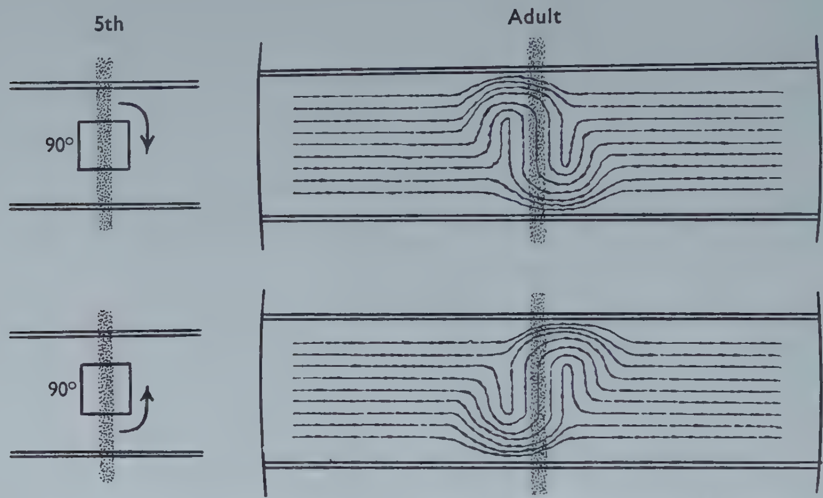
Text-fig. 9. The effect upon the adult cuticle of rotating a square of integument through 90° either clockwise or anti-clockwise (cf. Pl. 8, fig. 7; Pl. 9, fig. 15).



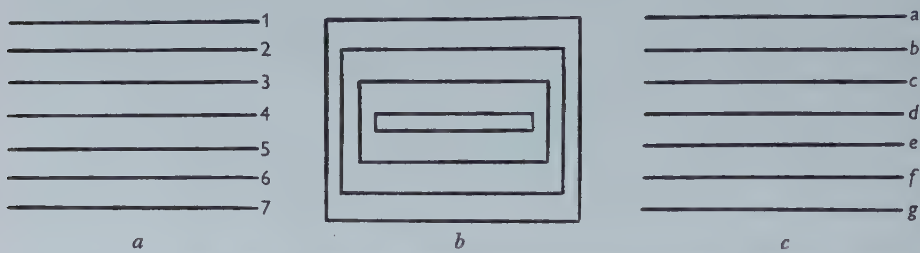
Text-fig. 10. The pattern which might have been expected in a square rotated through 90° if the cuticular ripples had been repaired by joining up the cut ends closest to one another.

A small square was cut in the centre of a tergite of one side, rotated through 90°, and replaced. The pattern formed in the adult depended upon the direction of rotation of the square, resembling the letter S or its mirror image (Text-fig. 9; Pl. 8, fig. 7; Pl. 9, fig. 15). The ripples upon the square and the surrounding cuticle joined up so that those of the square were continuous with the transverse pattern in the same direction as they would have been without rotation. Without the 'handedness' implicit in a gradient, a pattern of the form seen in Text-fig. 10 might have been expected. It is as if the cut edges of the square and hole retained an affinity for one another, the repair of the pattern tending to minimize the 90° displacement. This effect is not connected with a mesial-lateral gradient. A similar

result is obtained if the square is cut and rotated in the mid-line (Text-fig. 11; Pl. 9, figs. 16, 17). A 'handedness' does not necessarily imply a transverse quality. It can result from any axial gradient.



Text-fig. 11. The effect upon the adult cuticle of rotating squares of integument through 90° clockwise and anti-clockwise in the mid-line where mesial-lateral effects should be symmetrical (cf. Pl. 9, figs. 16, 17).



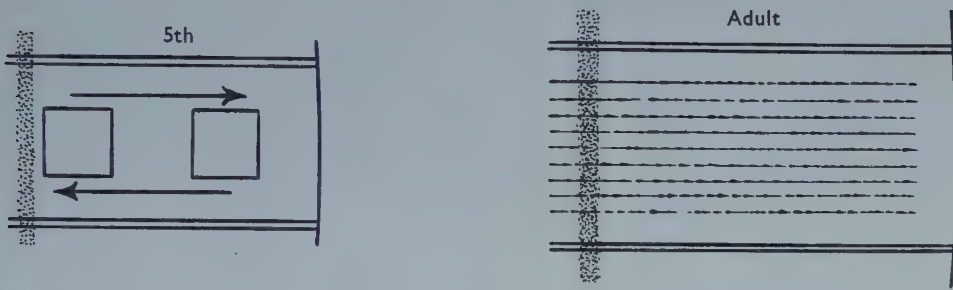
Text-fig. 12. The forms which the gradient might take within each segment: (*a*) an axial gradient of greatest intensity either anteriorly or posteriorly, (*b*) a contoured gradient with the lateral limits set close to the margins and not necessarily symmetrical from the centre as drawn here, (*c*) specific lateral affinity without quantitative form.

The form of the gradient

The gradient could take one of several forms, the only necessary constant being the similarity of properties in the transverse direction. For example, it could be a quantitative change from the anterior to the posterior (Text-fig. 12*a*), or the segment might be contoured (Text-fig. 12*b*) simulating an axial gradient from the centre. It need not involve any quantitative change. Each level in the axis might have a specific qualitative affinity for its lateral neighbours (Text-fig. 12*c*). Equally well a quantitative change from anterior to posterior could itself result in a specific lateral affinity.

If the gradient is contoured as in Text-fig. 12*b*, the mesial-lateral effects at the sides would not have been noticed because experiments have only been performed

in the centre of the segment. Two squares were cut and interchanged as in Text-fig. 13. The pattern in the adult was not disturbed. Thus if the gradient is contoured the lateral limits must be very close to the edge of the segment. The possibility of a gradient from the centre of the segment has been tested in another way. If there is such a centre then a square rotated through 180° about it should not disturb the symmetry of the pattern. A number of squares were cut at slightly different levels in the axis and rotated through 180° in an attempt to confirm this. A discontinuity pattern always resulted. Thus there is no evidence for a gradient from the centre of the segment.



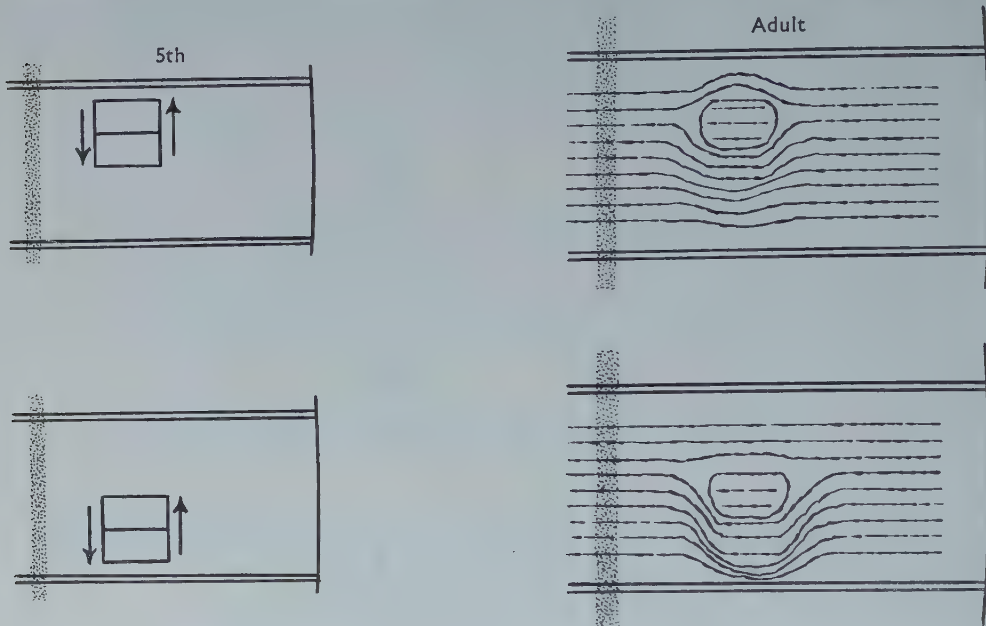
Text-fig. 13. The effect upon the adult cuticle of interchanging squares of similar transverse level in the larva.

This conclusion has been confirmed in another way. A quantitative difference from the centre might be expected to affect the facility with which continuity is established in axially displaced cuticle. Pairs of rectangles were cut and interchanged as in Text-fig. 14. With a greater lateral affinity in the centre, the rectangle farthest from the intersegmental membrane might be expected to be the one isolated in the discontinuity pattern. If the converse is true, with the greatest lateral affinity away from the centre, the rectangle towards the edge should be isolated. In a head-to-tail axial gradient the isolated rectangle should be consistently anterior or posterior independently of its position relative to the intersegmental membrane.

Pl. 10, figs. 18, 21 show the result. Except in a few instances where double discontinuity patterns formed, the rectangle from the posterior part of the segment is the one isolated. The anterior region of the segment shows dominance over the posterior region in its ability to restore pattern continuity after displacement. The double discontinuity patterns are probably caused by the migration of host cells across the wounded cut surface between the two rectangles, producing a result similar to the experiment described in Text-fig. 15.

These experiments suggested that the gradient might be an axial one as in Text-fig. 12*a*, one of its properties being the greater facility with which pattern continuity is restored anteriorly. This being so the transverse line of cells in the centre of a square rotated through 180° remains in the same position relative to the surrounding cuticle. A pair of discontinuity patterns would therefore be expected upon each side of this transverse level. In the earlier experiments small squares

were cut and this double pattern escaped notice, but in larger squares or in rectangles with the long axis in the axis of the animal this double pattern does indeed occur (Pl. 10, fig. 19; Pl. 9, figs. 10, 11), but the two patterns are never of equal size, the posterior one always being difficult to distinguish. This could be interpreted to mean that some of the cells of the square which are more anterior in origin tend to establish continuity with the edges of the hole, and in doing so isolate the major



Text-fig. 14. The effect upon the adult cuticle of interchanging adjacent rectangles of integument in varying positions in the axis within each segment (cf. Pl. 10, figs. 18, 21).

part of the square, forcing it into a discontinuity pattern. The remainder of the anterior cells are lost in small squares, or in large squares go to make the much smaller and more irregular posterior discontinuity pattern. This interpretation would be in agreement with the conclusion above that the most anterior parts of the segment have the greatest capacity for establishing pattern continuity. This description in terms of a segmentally repeated axial gradient is taken further in the discussion.

An interpretation of the discontinuity pattern

When a piece of cuticle is rotated through 90° the ripples in the adult do not end blindly but unite with the undisturbed transverse pattern. The cells responsible for the pattern have a capacity for restoring their relative positions to one another as far as the disturbance of the operation allows. The same effect is noticeable in the cuticle surrounding a square which has been rotated through 180°. The ripples at the same level on each side of the square are joined to one another by ripples deflected round the disturbed region. In experiments interchanging two pieces of cuticle in the axis transverse continuity is maintained through one of the pieces.

The ripples from the cut edges of the hole are deflected to unite with the cuticle which used to be at the same transverse level. It is evident from all these experiments that the cells behave in such a way that transverse continuity is maintained with cells similar in position in the axis. Now in two of the experiments mentioned above a piece of cuticle has been prevented from establishing this continuity. In the squares rotated through 180° the surrounding cuticle has maintained continuity round the square. In the axially displaced cuticle continuity could only be maintained with both rectangles if ripples could cross one another. The absence of such a pattern indicates that the epithelium cannot have a dual potentiality, it has no capacity for joining up with two levels in the axis. These pieces of cuticle isolated from the normal transverse continuity presumably retain an unsatisfied capacity for joining up with the appropriate level. Continuity will be satisfied least at the cut edges of the square. It has been suggested that the most anterior levels satisfy their capacity for continuity by uniting with one another round the square. A detailed examination of the patterns gives this impression. In the patterns induced by rotating through 180° the continuity is restored anteriorly. The posterior part of the square, anterior in origin, remains least disturbed but the edges extend in narrow ridges anteriorly, sealing off that part of the square which is posterior in origin (Pl. 10, figs. 19, 22). In the patterns induced by axial displacement the ridges on the anterior part of the square are least disturbed and continuity is restored posteriorly (Pl. 10, fig. 23). If this is the correct interpretation it might be expected that if the operation were performed in an earlier instar the adult would show a more concentric type of pattern with fewer transverse ripples ending freely. Squares were cut in 3rd-, 4th- and 5th-instar nymphs and rotated through 180°. The patterns in the adult showed a progressive trend from transverse to concentric type with the number of instars which had elapsed since the operation (Pl. 10, fig. 25).

It may be concluded that the discontinuity pattern is the result of the capacity of the epithelium for uniting cells of the same level in the axis.

Ripple orientation and the control of growth

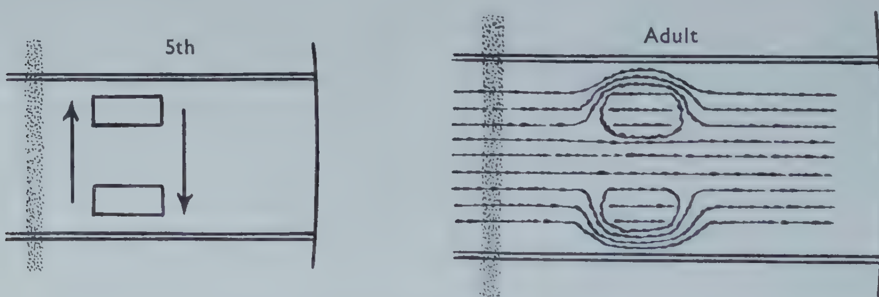
It has been assumed that the ripple pattern of the cuticle is caused by, and reflects, some similarly oriented mechanism within the cells of the epithelium. The question arises whether any other property of the cells obeys similar rules. In the tracheal system it has been shown that epithelial continuity is necessary for the quantitative control of growth but not for the normal sequence of events at moulting (Locke, 1958*b*). The tracheal epithelium is polarized with respect to this control: a trachea cut from its tissue connexions moults but does not grow normally in diameter. If the gradient in the axis of the tergites is homologous with the polarity of growth control in the tracheal system, the cuticle isolated in a discontinuity pattern might be expected to behave like a trachea isolated from its tissue connexions, moulting normally but without the usual increase in size.

The effects of isolation in a discontinuity pattern upon growth in area have been followed making use of the darkly pigmented sternal cuticle as a marker. The sternal cuticle also differs from the tergal in retaining bristles in the adult, but it

has similar transverse ripple marks, and grafts to the tergites take satisfactorily. Squares were cut from the centre of the sternites in 3rd-instar larvae and implanted in similar positions in the tergites. Some were rotated through 180° to induce the discontinuity pattern, others were implanted with normal orientation as controls. The changes in area could be followed by examining the exuviae. The difference between the isolated cuticle and the normally oriented transplant were most marked in the adult (Pl. 10, figs. 24, 26). In the rotated squares the transplant was reduced to a few bristles with no pigmented cuticle. In those with normal orientation the transplant was marked by an oval of pigment with normally spaced and oriented bristles. These results are in agreement with the hypothesis derived from a study of growth in tracheae that epithelial continuity is necessary for the quantitative control of growth. The epithelial continuity must be of a particular kind, being part of an axial gradient in the normal tergite.

Further properties of the orienting mechanism

It has been shown that the tergal epithelium responds to various disturbances by restoring the transverse continuity of the cuticular pattern. This could be described as an affinity between the cells at each level in the axis, the strength of the affinity being greatest anteriorly. The patterns obtained in some experiments point also to an interaction between cells of different axial levels as well as to the affinity between cells of the same level.



Text-fig. 15. The effect upon the adult cuticle of interchanging non-adjacent rectangles of integument in the axis (cf. Pl. 10, fig. 20).

Two rectangles were cut from the same tergite, one towards the anterior margin, and one towards the posterior margin, and interchanged as in Text-fig. 15. In the adult each showed a discontinuity pattern, being isolated from its own level by the undisturbed central strip of cuticle. The surrounding ripples were not evenly distributed anteriorly and posteriorly to the rectangle as in squares rotated through 180°, but almost entirely to the side between the transplant and the intersegmental membrane (Pl. 10, fig. 20). A similar effect was noticeable in earlier experiments (Pl. 9, figs. 13, 14). The pattern is easily explained if there is a reaction between the graft and the host varying with the difference in level in the axis. In Text-fig. 15 the rectangle close to the anterior intersegmental membrane has come from a posterior region of the segment and the surrounding pattern is stimulated to move

anterior to it. The rectangle close to the posterior intersegmental membrane has come from the anterior region of the segment and the surrounding pattern is stimulated to move posterior to it. The discontinuity pattern of an isolated square is normally brought about by the anterior regions uniting with one another posteriorly. In the displaced rectangle from the posterior region in Text-fig. 8, this results in the graft and host maintaining continuity in opposite directions and a clear-cut discontinuity pattern is formed. In the displaced rectangle from the anterior region both graft and host establish continuity posteriorly and the discontinuity is much less distinct. But the direction of orientation of a displaced square is much less important in its effect upon the surrounding cuticle than the difference in level in the axis. The host pattern is not markedly altered if the experiment described in Text-fig. 8 is repeated but with the squares rotated through 180°. Nothing can yet be inferred about the cellular mechanism of this response, it could be due to the migration of cells or it could be a transference of information from one cell to the next, but it serves to confirm the existence of an axial gradient.

DISCUSSION

Earlier work

The problem posed by oriented cuticular patterns has been discussed by Wigglesworth (1954*b*) with particular reference to the orientation of the bristle-bearing plaques. He had noticed (1940) that plaques regenerating over a burned area had normal orientation and that the plaques on rotated cuticle retained their original orientation. He therefore concluded that some sort of orientation existed within the cytoplasm of the cells. He says: 'These observations point to the existence of some kind of "cytoskeleton" within the undetermined cell, which defines the anterior-posterior axis and controls the mutual relations of the daughter cells...and in this way controls the orientation of the resultant structures.' The observations upon the mechanism of orientation of the ripple pattern confirm and amplify this conclusion.

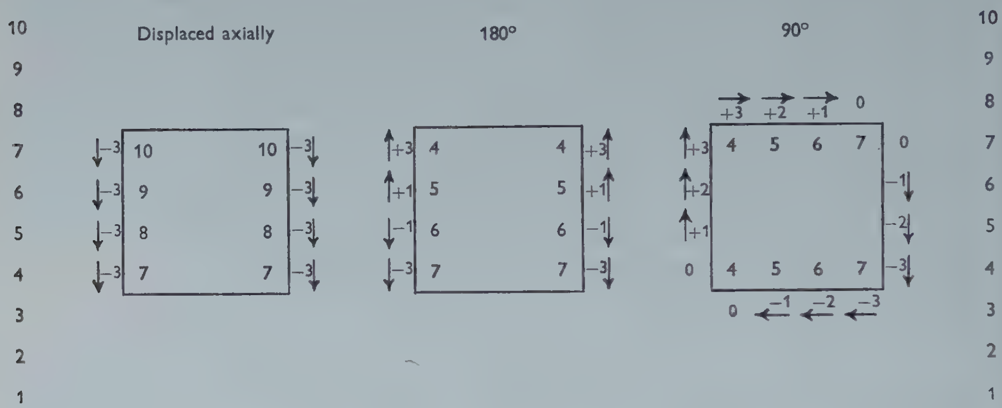
The problem has also been studied experimentally by Piepho (1955) using the oriented bristles in *Galleria* larvae. He described an interaction at the boundaries of rotated cuticle where the orientations change. His results might also be described in terms of a gradient.

The cuticular pattern

The adult cuticular pattern gives no indication of the gradient in the epithelium inferred from the experiments. It has an axis of symmetry requiring at most a polarity for its orientation. The experiments neither confirm nor deny the hypothesis of expansion with an orienting stress and ripple-like buckling; they only show that any such postulated stress must operate at the cellular level. This could be due to non-uniform restraint as in tracheae or to unequal expansion. The behaviour of the epithelium—the facility with which lateral continuity of the pattern is restored—might well reflect a difference in mechanical properties in different directions. Put in its crudest way the cell membranes might be stretched by the side-to-side continuity, making them more resistant to buckling in that direction. This approach will have to await future work upon the cellular basis of the pattern.

The gradient concept

The term axial gradient has been introduced to describe the mechanism of orientation of the adult pattern of cuticular ridges. It is merely a shorthand description of the results of the experiments, representing no advance in itself. Its usefulness is more easily seen if the gradient is given numerical values (Text-fig. 16).



Text-fig. 16. A linear gradient is indicated by the numbers 10–1. The differences in value between the square and the host gradient are shown in small numbers. The direction in which the host ripples maintain continuity is indicated by arrows. Three typical experiments are shown in the diagram. In each the negative or positive value of the gradient difference agrees precisely with the direction in which continuity is maintained (cf. Text-figs. 2, 8, 9).

In a square displaced axially from an anterior region there is a consistent difference in level along the lateral margins of the square, and the host ripple lines all unite posteriorly to it. In a square rotated through 180° the difference in level varies with opposite sign from a neutral region at the centre of each lateral margin. In agreement with this the host pattern divides and unites equally anteriorly and posteriorly. In a square rotated through 90° there are two corners opposite to one another (the corners varying with the direction of rotation) where there is no difference in level. The S-shaped pattern results by uniting the ripples along the sides of the square in the direction determined by the sign of the difference in levels. In all three examples the host epithelium reacts to that of the square according to the level of the latter, moving anterior or posterior to it until the levels are similar and the pattern is reunited. Evidence will be presented in a future paper that this is due to the migration of cells. If this were all, the term gradient might be inapplicable, since the results could be accounted for without any quantitative axial change. There is no evidence that the degree of interaction varies quantitatively as the numbers in Text-fig. 16 might suggest. Qualitative recognition of difference of level, + or −, between cells would suffice to account for the capacity of similar levels to unite. But a gradient is suggested in another way. The anterior cells show dominance over the posterior cells in restoring pattern continuity. The concentric pattern in any piece of cuticle showing discontinuity is formed by the

anterior cells uniting posteriorly however the discontinuity has been induced. In adjacent rectangles interchanged in the axis continuity is established through the cuticle which is anterior in origin. The recognition of level and the anterior dominance could be considered as separate properties of the epithelium. For the moment it is convenient to consider both as being due to a gradient, but this may not prove a satisfactory description for long.

The most important property of the gradient then is the behaviour of the cells resulting in continuity of the ripples of the same level.

The gradient and the control of growth

Isolated pieces of cuticle may have satisfied their capacity for uniting with cells of the same axial level by forming a concentric pattern, but they fail to grow harmoniously with the rest of the animal. Satisfaction of the capacity for completing the pattern is alone insufficient to induce normal growth, the cuticle must also be in the gradient of the whole segment. The quantitative control of growth is therefore not coincident with the behaviour of the cells described as a gradient. If the amount of growth depended upon a blood-borne factor alone, the converse might have been expected. That this is not so implies that in the normal animal the cells receive information about the growth to be made in some other way—presumably through the epithelium as in tracheae. The cuticle in a discontinuity pattern differs from normal cuticle in that the ridges 'do not go anywhere', they unite with themselves. If this is the cause of the failure of controlled growth it could mean that in the continuity of the normal segment the cells receive information from somewhere in the direction of the gradient. This may be the real significance of the cells maintaining contact with their own level. As revealed by the abnormal conditions of the experiments, the gradient appears as a curiosity. To the animal it may be a mechanism for maintaining the cells in a preferred order for the transport of growth stimuli. The source of these growth stimuli will be explored in a future paper.

SUMMARY

1. The cuticle on the abdominal tergites of *Rhodnius* larvae has a uniform pattern of stellate pleats. In the adult this pattern is replaced by an oriented one of transverse ridges resembling greatly elongated pleats.

2. Neither pattern results from the disposition of cells each with the potentiality for forming only part of it. The simplicity of the patterns suggests some simple mechanical cause operating over a large area.

3. The effect upon the adult of altering the orientation and relative position of pieces of larval integument has been used to study the mechanism of orientation of the pattern.

4. The orientation of the adult pattern is mediated through the epithelium, since rotation of the larval integument causes corresponding disturbances in the adult pattern.

5. The epithelium has a capacity for maintaining transverse continuity within

similar levels in the axis. There is an axial gradient within each segment, the anterior showing greatest facility in maintaining continuity.

6. Pieces of transplanted integument unable to restore their continuity with the whole animal show a concentric pattern of ridges, as if the capacity for continuity had been satisfied by each level joining up with itself.

7. Such isolated pieces of integument moult but fail to grow normally, suggesting that continuity in the axial gradient of the whole animal is necessary for the quantitative control of growth.

8. Pieces of integument from different axial levels within a segment when juxtaposed influence the direction of displacement of the pattern according to their level. A transplant from the anterior stimulates the host ridges to maintain continuity posterior to it, and conversely.

I am grateful to Mr G. L. Underwood for reading the manuscript and to Mr M. Gill for assistance with the photography. I also thank Professor V. B. Wigglesworth for some helpful comments and for referring me to the work of Piepho.

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EXPLANATION OF PLATES

PLATE 8

All figures except 4 and 5 are unstained whole mounts of *Rhodnius* cuticle taken with a phase contrast microscope and oriented so that the head end is at the top of each figure.

Fig. 1. 5th-instar larval cuticle showing plaques and unorientated stellate pleats.

Fig. 2. The centre of abdominal segment 2 of one side in an adult showing the transverse ripples.

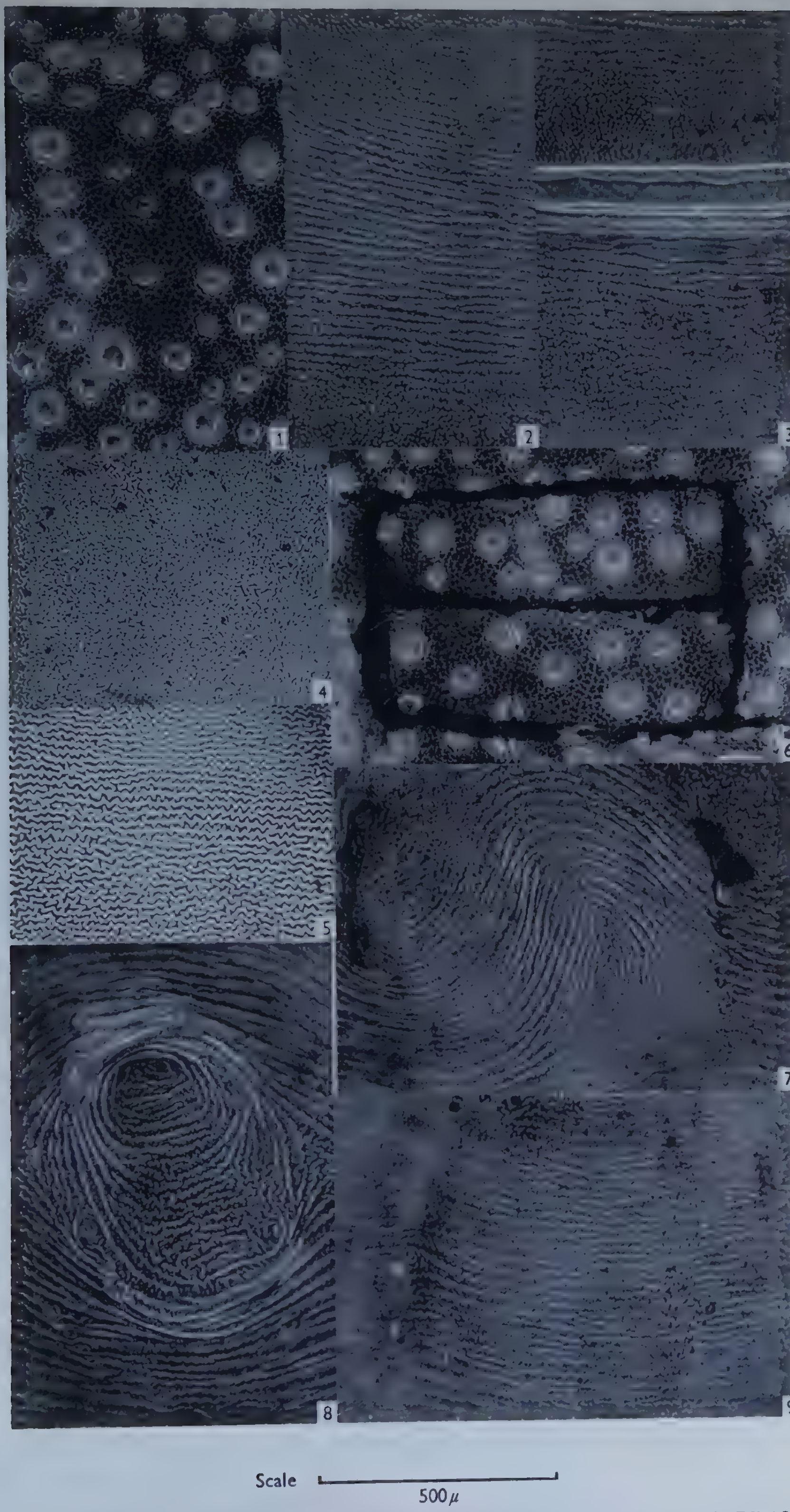
Fig. 3. The intersegmental membrane between segments 2 and 3.

Fig. 4. Random buckling in a rubber and gelatin model of larval cuticle.

Fig. 5. Oriented buckling produced in another model by stretching from side to side.

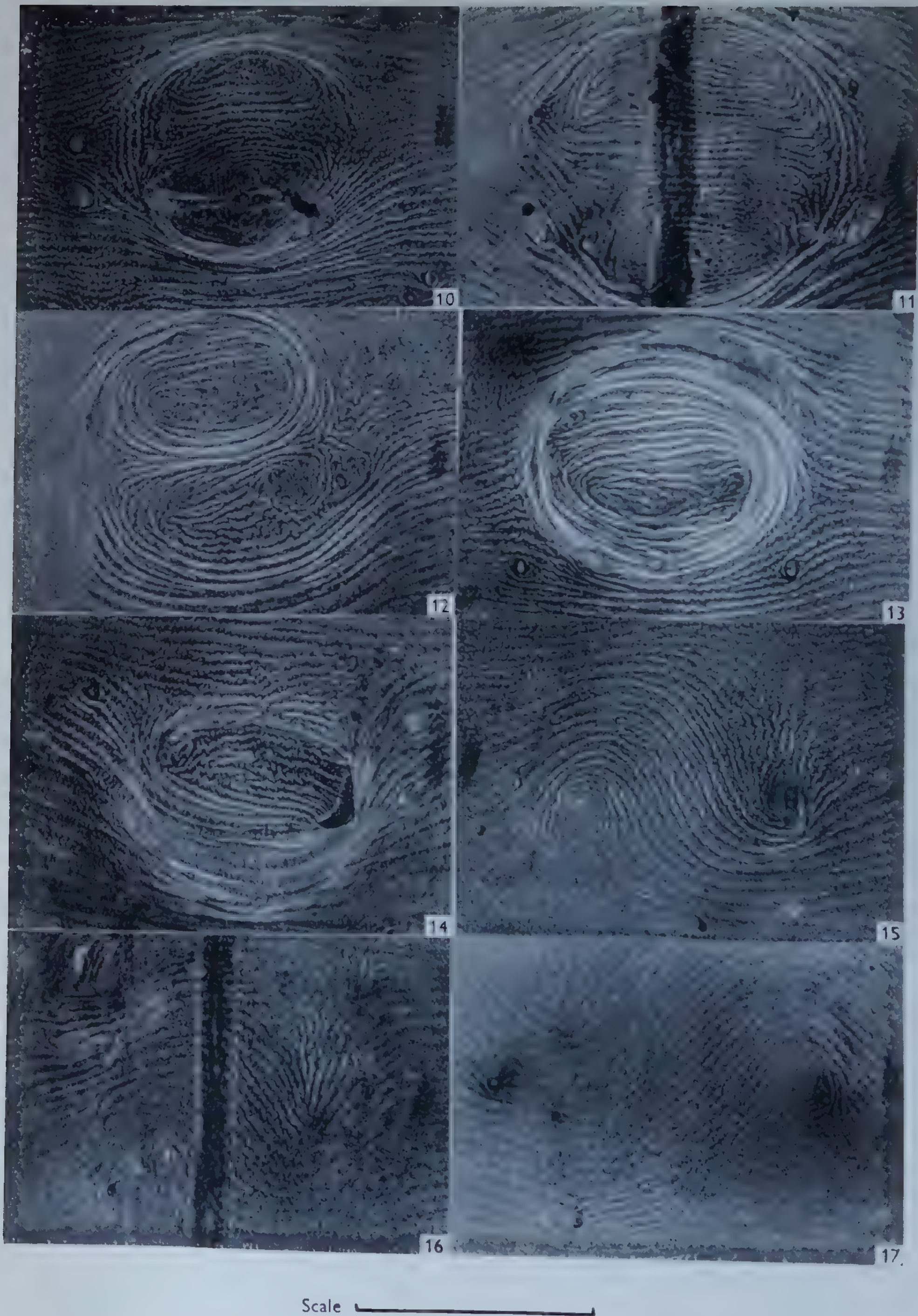
Fig. 6. The 5th-instar exuvium, showing an operation in which the two rectangles of cuticle have been interchanged (cf. Text-fig. 6).

Fig. 7. The effect upon the adult of rotating a square of integument in the larva 90° anti-clockwise (cf. Text-figs. 1, 9).



LOCKE—THE CUTICULAR PATTERN IN AN INSECT, *RHODNIUS PROLIXUS* STÅL

(*Facing p.* 476)



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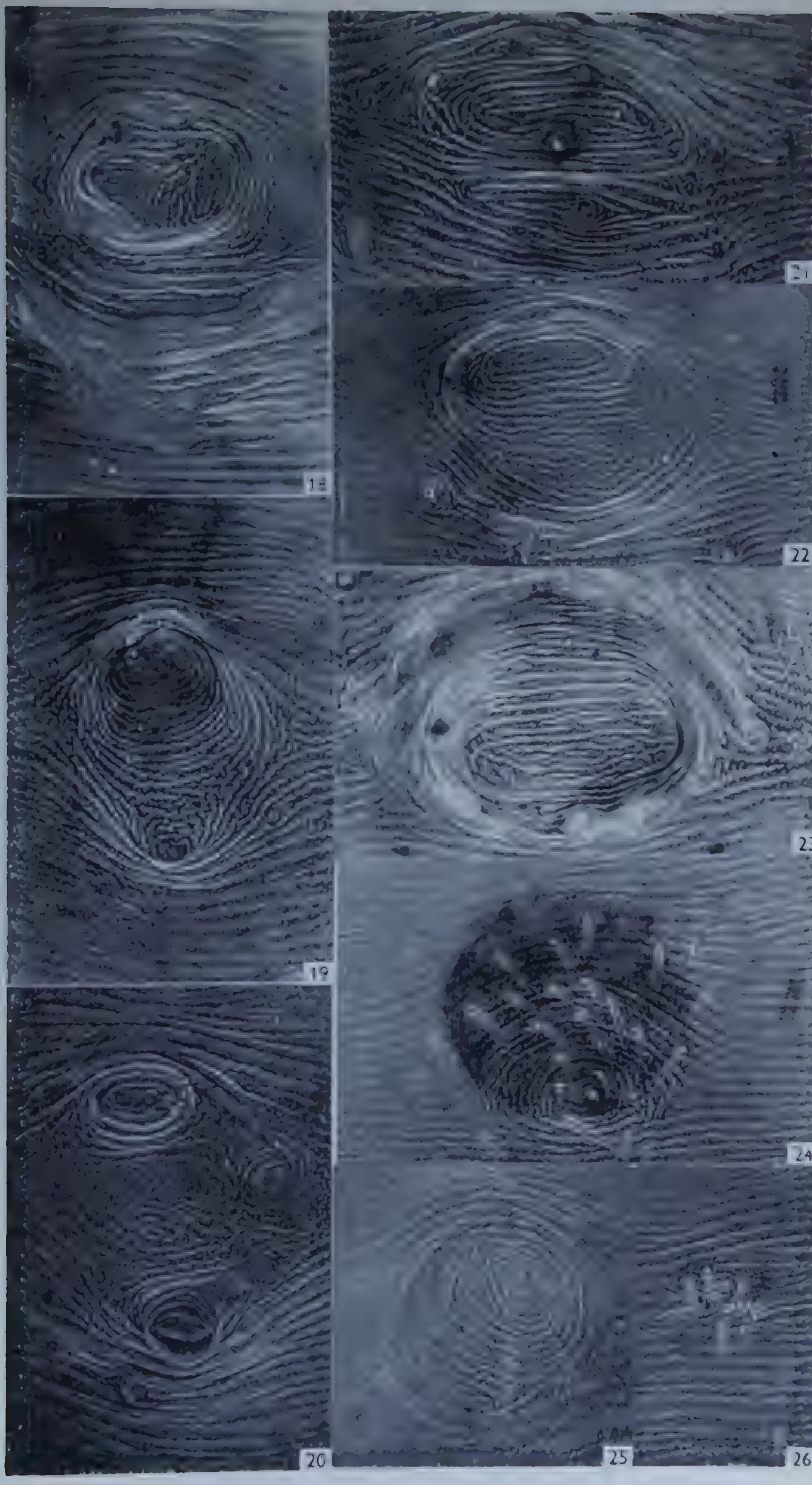

Scale 500 μ

LOCKE—THE CUTICULAR PATTERN IN AN INSECT, *RHODNIUS PROLIXUS* STÅL

Fig. 8. The adult pattern when a square of cuticle has been rotated through 180° in the larva (cf. Text-fig. 2).

Fig. 9. The effect of interchanging squares of cuticle in symmetrical positions upon either side of the mid-line. The transplant of one side only is shown (cf. Text-fig. 3).

PLATE 9

All the figures are phase contrast photomicrographs of the cuticular pattern in adult *Rhodnius* showing the effects of operations upon the larvae.

Fig. 10. The effect of reversing the anterior-posterior direction of a square of cuticle in the larva while leaving the mesial-lateral direction unchanged (cf. Text-fig. 4).

Fig. 11. The effect of reversing the anterior-posterior direction in the mid-line where mesial-lateral effects should be symmetrical (cf. Text-fig. 5).

Fig. 12. The effect of interchanging adjacent rectangles of integument in the axis (cf. Text-fig. 6).

Fig. 13. A square of cuticle has been transplanted from the posterior part of one segment to the anterior part of the segment shown (cf. Text-fig. 8).

Fig. 14. A square of cuticle has been transplanted from the anterior part of one segment to the posterior part of the segment shown (cf. Text-fig. 8).

Fig. 15. The effect of rotating a square of larval integument 90° clockwise (cf. Text-figs. 1, 9).

Fig. 16. The effect of rotating squares of larval integument clockwise through 90° in the mid-line where mesial-lateral effects should be symmetrical (cf. Text-fig. 11).

Fig. 17. The effect of rotating a square of larval integument through 90° anti-clockwise in the mid-line where mesial-lateral effects should be symmetrical (cf. Text-fig. 11).

PLATE 10

All the figures are phase contrast photomicrographs of the cuticular pattern in adult *Rhodnius* showing the effects of operations upon the larvae.

Fig. 18. Two adjacent rectangles have been interchanged in the axis in the anterior half of a segment. The rectangle posterior in origin has formed a discontinuity pattern (cf. Text-fig. 14).

Fig. 19. The effect of rotating through 180° a rectangle with its long axis in the axis of the animal. A partial double discontinuity pattern has formed.

Fig. 20. Two rectangles separated by a strip of untouched cuticle have been interchanged (cf. Text-fig. 15).

Fig. 21. Two adjacent rectangles in the posterior half of a segment have been interchanged in the axis. The rectangle posterior in origin has formed a discontinuity pattern (cf. Text-fig. 14).

Fig. 22. A discontinuity pattern obtained by rotating a square through 180° (cf. Text-fig. 2).

Fig. 23. A discontinuity pattern obtained by displacing a square from a more posterior position in the axis to its present one in the anterior half of a segment (cf. Text-fig. 8).

Fig. 24. The remains of a square of sternal cuticle with normal orientation taken from the centre of a segment in the 3rd instar. The sternal cuticle is distinguished by its darker pigmentation and bristles (cf. Fig. 26).

Fig. 25. A more concentric discontinuity pattern obtained by rotating a square of integument through 180° in the 4th instar.

Fig. 26. The remains of a square of sternal cuticle taken from the centre of a segment in the 3rd instar and rotated through 180°. No pigmented cuticle and few bristles remain (cf. Fig. 24).

PHYSIOLOGICAL MECHANISM OF NEMATOCYST RESPONSES IN SEA-ANEMONE

VII. EXTRUSION OF RESTING CNIDAE—ITS NATURE AND ITS POSSIBLE BEARING ON THE NORMAL NETTLING RESPONSE*

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(*Received* 22 *December* 1958)

The nettling response of coelenterate nematocysts (nettling capsules or cnidae), as it occurs in a natural manner, often involves the capsules themselves being cast off from the animal's body surface. The latter event might be taken as a simple mechanical effect of the tension which is exerted upon the stinging thread when this becomes attached to the struggling victim. Parker & van Alstyne (1932), however, tend to see in it an active participation of the organism. They showed that a solution of ether in sea water caused the cnidae to go out of the acontia of *Metridium* with their threads still undischarged, and suggested it to be an agent imitating that 'final step in the nettling operation'.

Similar phenomena had already been described by some earlier workers. Thus, Abric (1904) showed that emission of undischarged cnidae took place from *Actinia*, *Tealia*, etc., through the action of 'aqueous thionine'. He described the active appearance of that event by stating 'ils [undischarged cnidae] ne sont pas seulement laissés libre par les nématoblastes, mais projetés par lui'. Glaser & Sparrow (1909) introduced the term 'extrusion' to designate the event in question. According to them, the term should be clearly distinguished from that representing another feature of cnida action, the 'explosion' (discharge or eversion of stinging thread). More recently, Pantin (1942) has reported anaesthetizability by magnesium ions of the extrusion response, which he found to be elicited by various agencies on the tentacle of *Anemonia sulcata*.

The present writer (Yanagita, 1943), too, has found that salt-free media, like distilled water or glycerine (or glucose) solutions, afforded remarkable instances of cnida extrusion from the acontia of *Diadumene luciae*. The cnidae thus extruded were unexploded and still reactive. The phenomenon, its nature being still unknown at that time, has since been used as an excellent means of obtaining isolated cnidae in quantity for experimental uses. Visual impressions of the process of extrusion were really suggestive of an active physiological response as mentioned by Abric. They differed remarkably from impressions of the passive liberation of cnidae caused by many cytolytic agents. Indeed, in the case of the extrusion produced in isotonic glycerine or glucose solution the acontial structure with its ciliated surfaces

* Supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education.

seemed to remain intact for a long time after the extrusion. It was therefore a matter of interest to inquire whether the extrusion actually represents an active response of the organism and, further, what significance it has in the physiology of cnidae.

It is the aim of the present work (1) to analyse the exact working conditions of extrusion-inducing 'stimuli', (2) to ascertain whether any anaesthetic is really effective in preventing the reaction, and finally (3) to consider the nature and mechanism of the extrusion as well as its possible role in the normal nettling response of cnidae.

MATERIAL AND METHODS

Materials used were acontial filaments cut off into sea water from *Diadumene luciae*. The type of cnidae dealt with in the present study was exclusively the 'microbasic *p*-mastigophore' in the nomenclature of Weill (1934) as supplemented by Carlgren (1940),* as has been the case in all of the previous papers.

A piece of acontium, usually about 1·5 cm. in length, was mounted with a small amount of sea water on a glass slide, and the medium was replaced with the experimental solution which was to be examined under the microscope for its extrusion-eliciting or anaesthetizing effect.

Different methods of mounting and replacing the medium were used as follows, singly or jointly according to the occasion. (1) Hollow-slide method, in which the acontium was stretched in sea water between two parallel strips of filter-paper somewhat aside from the central hollow of a hollow-slide, and a coverslip was put over them. The medium could be renewed freely by suction from the hollow part of the slide, the new medium entering at the other end of the cover. (2) Simple stretched mount on an ordinary slide, where both ends of the acontium were attached directly on the dry surface of glass, its middle portion being bathed in a small pool of sea water. (3) Simple free mount in a drop of sea water. In this case the acontium usually became coiled up and rotated slowly under the action of its cilia. A coverslip was not used in the latter two methods, and the exchange of medium was effected by simply removing the previous one by means of a finely drawn pipette and then adding the new one.

A very rapid and thorough change of the medium was sometimes required, particularly for a sufficient amount of extrusion to be brought about by a salt-poor medium. Such could be accomplished through lifting the acontium out of sea water by one end caught on a needle tip and mounting it directly in the test solution. No appreciable response of the acontium was elicited by such mechanical handling itself. The mounting was done either by method 2 or by method 3 described above.

* The acontium of *Diadumene luciae* contains also far smaller 'basitrichous isorhizas' (see Hand, 1955), which were found to show behaviour different from that of the mastigophores either as to extrusion or discharge; they were not extruded in a salt-free medium.

RESULTS

(1) *Effect of dilution of sea water*

First, the effectiveness of distilled water in causing extrusion from *Diadumene* acontium was examined quantitatively. When sea water progressively diluted with distilled (or de-ionized) water (unbuffered) was tested, in the ways described above, for its extrusion-inducing capacity, it was found that the extrusion first set in at a dilution of as much as twenty times (osmotically equivalent to about M/40 NaCl). The maximum effect was practically attained at dilution of fifty times. A representative set of data, obtained by the use of acontia from a single anemone, is shown diagrammatically in Fig. 1. Each point was derived from a single acontium (1·5 cm. in length) which was transferred directly (see above) to the test medium and kept there for the short duration indicated by the abscissa. The number of the cnidae extruded and left behind was counted after removal of the acontium. It may also be seen from the diagram that the reaction, already apparent within ten seconds of exposure to each of the effective media, reached its full extent in about 30 sec.

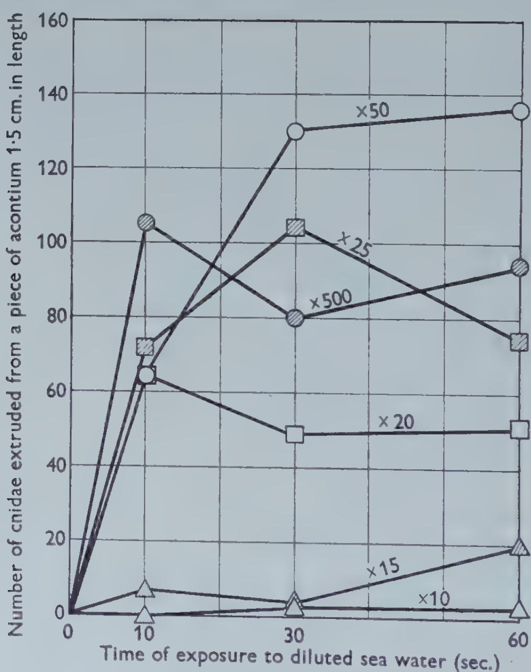

Fig. 1. Time courses of cnida extrusion in different dilutions (× 10–× 500) of sea water with distilled water. Temp. 19·0° C.

As it was known that marked extrusion took place also in pure (aqueous) solutions of glycerine or of glucose, even in concentration isotonic with sea water (1 M), it seemed likely that the efficacy of distilled water or of diluted sea water was not due to its hypotonicity. It was suggested that a lowered concentration of the sea

water or of some of its components might have been responsible for the present effects. In order to confirm this point, different dilutions of sea water by means of 1 M glycerine solution were examined for their extrusion-inducing capacity, and it was found that the corresponding degrees of dilution actually induced the cnida extrusion in this case also.

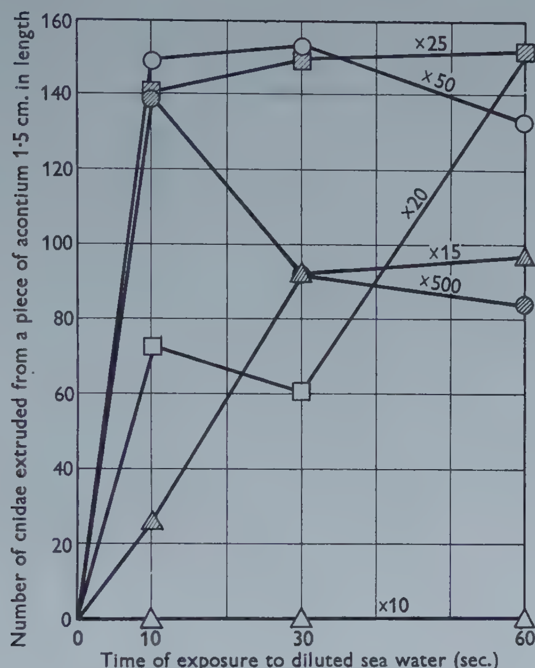

Fig. 2. Time courses of cnida extrusion in different dilutions (× 10–× 500) of sea water with 1 M glycerine solution. Temp. 18·2° C.

Fig. 2 shows a set of data obtained from these experiments, which were of the same type as the foregoing ones except that 1 M glycerine solution (unbuffered) was used as the diluent for sea water. The results, though showing some irregularities, may be seen to be in rather good agreement with those obtained from the dilutions with distilled water. In both cases there is perhaps some indication that excessive dilution (500 times) tends to reduce the extrusion-inducing capacity, but any further inquiry into this point has not been attempted as yet (see also Fig. 4).

It has thus become clear that the extrusion-inducing effect depends only on the extent of dilution of sea water, irrespective of whether the dilution be made with pure water or with glycerine solution. Such a result might indicate the presence of some factor or chemical component of the natural sea water which actively *suppresses* the extrusion, so that on its diminution the extrusion would set in at once.

(2) *Visible features of the extrusion process*

As already mentioned, the extrusion of cnidae as it took place from *Diadumene* acontium in the media described above was such as to give a visual impression of an active physiological response. The cnidae, which in the resting state are densely disposed within the acontial surface with their long axes perpendicular to the surface, now suddenly start to shoot straight forward into the external medium, until they usually leave the acontium entirely and are suspended in the medium close by. This is a brief, sometimes explosive, event which usually breaks out sporadically on the acontium and rapidly sweeps over the surface for some distance.

No visible change or movement on the part of the acontium has been noticed accompanying the extrusion reaction. Though coiling movements of acontial filaments (due to contraction of the longitudinal muscular bands) were sometimes observed to set in on transfer to an extrusion-inducing medium, this had apparently no correlation, either in time or in space, with the occurrence of extrusion. Further, there was no appreciable sign of breakage on the acontial surface at the point where the extrusion had taken place, even on examination with a high power. The acontium appeared to remain quite intact, showing active ciliary beat for a long time after the reaction. This was particularly the case when the experimental media were isotonic with sea water; under these conditions an endosmotic swelling of the tissue was prevented and the acontium was able to keep its normal appearance indefinitely, even after a considerable loss of its cnidae.

The extrusion reaction was graded in its intensity, i.e. in the relative amount of cnidae extruded, as can be seen from the results presented in the foregoing section. However, even in the case of maximum reaction, extrusion of all the cnidae possessed by the acontium was in no case to be obtained, a large number of cnidae usually still remaining in the acontium after the reaction.

In any of the effective media mentioned above the extruded cnidae remained unexploded indefinitely. This is in accord with the results already reported (Yanagita, 1943; Yanagita, 1959*a*) that distilled water or glycerine (and also glucose) solution had no power of provoking explosion in isolated cnidae of *Diadumene*. This cnida reaction therefore consisted purely of the extrusion process, thus affording excellent material for studying that process. It must be added here that the cnidae thus liberated in the medium from the acontium were still reactive for a long time, especially when a pure glycerine solution was used (Yanagita, 1951).

(3) *Effects of various salts*

In the hope of determining the component of sea water suppressing the extrusion as remarked in §2, isotonic salt solutions having a cation in common were used as the experimental media and tests were made on the presence or absence of extrusion from the acontium on transfer to each of those solutions. Various methods of mounting the acontium and of applying the experimental solution as described above were tried, including that of direct transfer of the acontium. Since K^+ as

well as NH_4^+ ions have been known to have an effect of 'exciting' the discharge mechanism of cnidae in the acontium (Yanagita, 1959*a*), the salts of both these ions were excluded from the present tests. The nature of their action on the acontium will be discussed in the latter part of this work, together with that of other kinds of discharge 'stimulants'. Mg^{2+} ions, on the other hand, were found to possess an 'anaesthetizing' effect on the extrusion response. Such action of Mg^{2+} will be dealt with under a separate heading.

The results obtained on the extrusion-suppressing effect of pure, isotonic (or hypotonic, for a reason which will be shown later) solutions of various Na salts are given in Table 1. pH values of the solutions used (unbuffered) were checked with indicators to be sure that they all fell within that range (pH 3–11) where H^+ or OH^- ions were without discharge-provoking action on isolated cnidae (Yanagita & Wada, 1953).

Table 1. *Extrusion-suppressing effect of anions in sodium salts*

(+ represents the presence, − the absence, of a suppressing effect, and ± an imperfect suppressing effect.)

SCN^-	+	($H_2PO_4^-$)	±
I^-	+	—	—
ClO_3^-	+	HPO_4^{2-}	±
(HCO_3^-)	+	(SO_3^{2-})	±
NO_3^-	+	SO_4^{2-}	−
Br^-	+	Tartrate^{2-}	−
Cl^-	+	(Oxalate^{2-})	−
Acetate$^-$	+	—	—
(Butyrate$^-$)	+	Citrate^{3-}	−

It may be seen from the table that Na salts of almost all the univalent anions which were tested actually possessed an action of suppressing the extrusion when applied in pure solution of sufficient concentration. They were able to replace natural sea water in that respect. Of the six plurivalent (five di- and one trivalent) anions, on the other hand, most were without such action. Thus, in the isotonic (M/3) solution of Na_2SO_4, the extrusion of cnidae took place with an intensity equal to, or even higher than, that which would be obtained in distilled water or in 1 M glycerine.

As for Li and Ca salts, only LiCl, $CaCl_2$, and $Ca(NO_3)_2$ could be included in the present tests. The results were the same as with the Na salts of the same anions; all of them were effective in suppressing the cnida extrusion. It is important here to note that a M/2 solution of choline chloride was tested in the same way and found to be an equally effective extrusion-suppressor. Similar results were obtained also with chlorides of mono-, di- and trimethylamine, and with chlorides of betaine, adrenaline and tetramethylammonium, as well as with tyramine hydrochloride.

It may be the simplest inference to be drawn from the present results that the extrusion-suppressing action is ascribable only to certain kinds of anions, whereas cations like Na^+, Li^+ or Ca^{2+} do not play any noticeable role. In Table 1, the anions are arranged in accordance with a generalized form of Hofmeister's lyotropic

series, except those enclosed in parentheses. The latter could not be included in the series due to the lack of published information, and have provisionally been given respective positions in the table in a quite arbitrary way. It may be pointed out from the table, that the members lacking extrusion-suppressing action are all gathered toward that end of the series which means stronger action in coagulating a hydrophilic colloid like egg albumin.

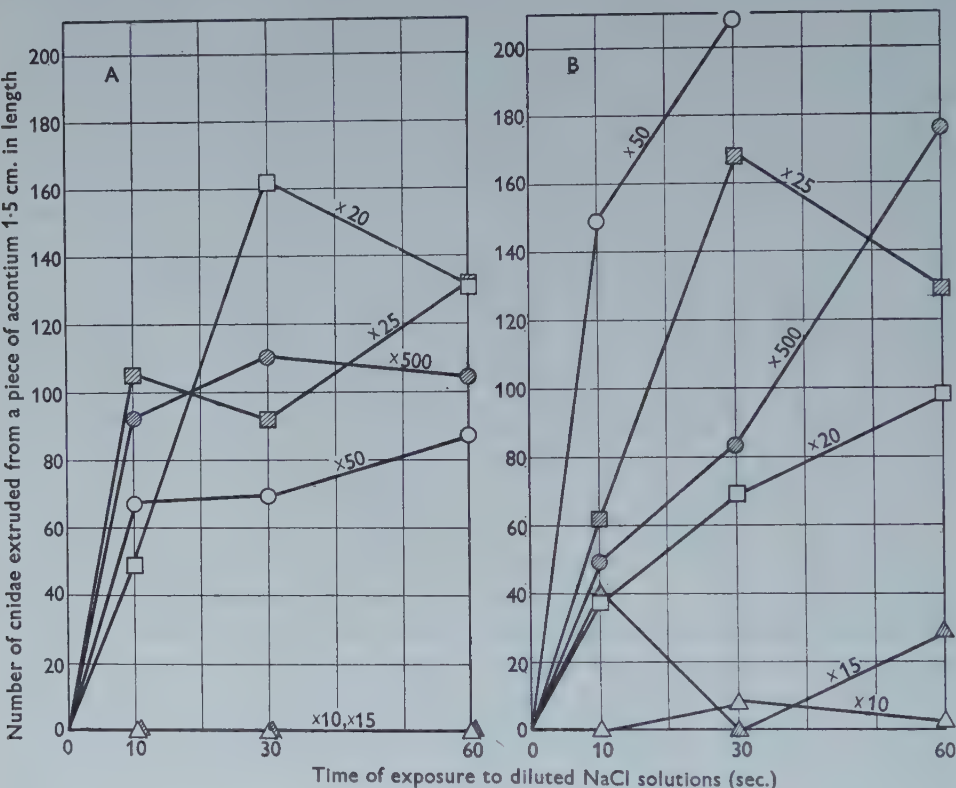

Fig. 3. Time courses of cnida extrusion in different dilutions (× 10 = M/20 to × 500 = M/1000) of M/2 NaCl solution with distilled water (A), and with 1 M glycerine solution (B). Temp. 18·5 to 19·0° C.

It now seems clear that the state of perpetual suppression of cnida extrusion, as we actually find it in acontia bathed in natural sea water, is ascribable to actions of its Cl^- and Br^- ions. The effect of a salt over a range of dilutions was quantitatively investigated only for the single instance of NaCl. Experiments of the type similar to those described in § 1 were carried out, this time using M/2 NaCl instead of sea water as the starting solution. A representative set of data obtained is given graphically in Fig. 3 A and B. It may be seen from the curves that it scarcely influences the result whether distilled water or 1 M glycerine was used as the diluent. Moreover, a comparison with the curves in Fig. 1 or Fig. 2 shows that M/2 NaCl is practically equivalent to sea water in extrusion-suppressing capacity over the whole

range of dilutions (Fig. 4).* Since a M/20 solution thus proved effective in suppressing the extrusion in the case of NaCl, and such was the case also with each Na salt given in the left-hand column of Table 1, the non-effectiveness of sulphate, tartrate, etc. may hardly be attributable to an insufficient concentration of the solution used, though no tests were made on concentrations higher than isotonic.

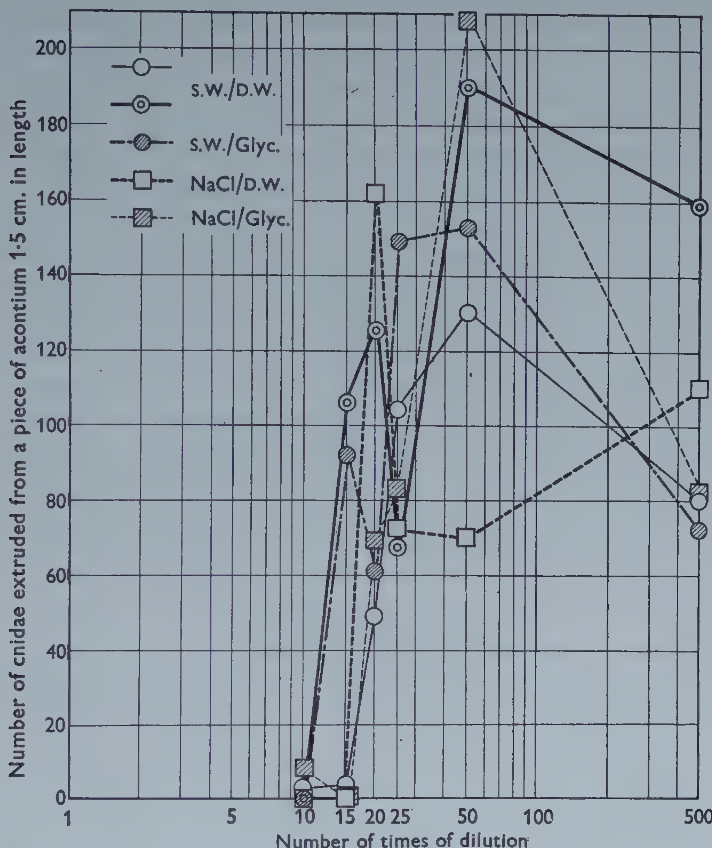

Fig. 4. Amount of cnida extrusion (in 30 sec.) as related to the dilution of sea water or of M/2 NaCl solution with distilled water or with 1 M glycerine. Re-drawn from the graphs presented in Figs. 1, 2 and 3 A and B, except for the plots in double circles. Temp. 18–19° C.

In the case of $NaNO_3$ it was found that the isotonic solution (M/2) provoked marked extrusion while perfect suppression was obtained only when it was diluted (with 1 M glycerine or with distilled water) to M/8 to M/16. In concentration as low as M/32 there was no longer such suppressing action and the extrusion reaction

* Published values of Dittmar's standard sea water for Cl^- and Br^- were, on re-calculation, 0·563 M and 0·00085 M, respectively. Added together, they correspond to a chlorimetric strength of M/1·776. Sea water which was used in the present experiments has actually given the value of M/1·793 on chlorimetry. Hence, M/1·8 instead of M/2·0 may have been the exact molarity of NaCl equivalent to sea water, provided one may assume the applicability of simple summation of effects between different anionic components.

reappeared. Since similar results have also been obtained with Na-thiocyanide, bicarbonate and iodide, they seemed to represent an effect characteristic of those anions which are situated at the upper extreme in the lyotropic series. These anions may be tentatively grouped as 'excessively effective' ones, in contrast to the 'adequately effective' anions like Cl^- and Br^-. On the contrary, anions at the opposite end of the series such as SO_4^{2-} were found to be 'absolutely ineffective' in that they showed no extrusion-suppressor effect in any further dilution.

As is reported in another paper (Yanagita, 1959*a*), many of the anions also possess direct action on *isolated* cnidae in provoking their explosion. However, the threshold concentrations for that action were, in general, definitely higher than those for the extrusion-suppressing action, so that those cnidae which were isolated through extrusion reaction into a salt-poor medium would necessarily remain unexploded forever, as mentioned in §2. Only the cnidae which became extruded in a concentrated solution of an anion belonging to the 'excessively effective' group, as well as those extruded in a concentrated solution of 'absolutely ineffective' anion (oxalate, citrate), would instantly get discharged under direct action of the respective anion. Sulphate or tartrate ion, on the other hand, being devoid not only of an extrusion-suppressing effect but also of the direct action on isolated cnidae (Yanagita, 1959*a*), extrusion produced in any concentration of such an anion would never lead to discharge, so that the whole efficacy of such salts much resembled that of a non-electrolyte like glycerine. As a matter of fact, M/3 Na_2SO_4 solution proved sometimes to be an extrusion-inducer even more effective than a salt-free medium. The reason for that is not clear at present.

(4) *Effects of anaesthetics*

Several sorts of chemicals commonly known as anaesthetics for excitable systems were examined in respect of their effect upon the reaction of cnida extrusion in the acontium of *Diadumene*.

(*a*) *Magnesium salts*. Pantin (1942) found that the solution of half 0·6M-$MgCl_2$ plus half sea water (his 'magnesium sea water') 'anaesthetized' all the cnida responses in *Anemonia* tentacle, including that of extrusion. The present writer used instead the mixture of M/3 $MgCl_2$ and natural sea water in equal volumes in the present test on *Diadumene* acontium, in order to maintain the isotonicity of the medium. When the acontium had been bathed in such Mg sea water for a few minutes prior to transfer to 1 M glycerine or M/3 Na_2SO_4 solution, the extrusion was found to be completely prevented. This effect of $MgCl_2$ was reversible; when the medium was again changed to normal sea water the acontium was found to recover its responsiveness to the glycerine or Na_2SO_4 solution in several minutes.

A similar effect was also obtained with $MgSO_4$. It was tested in different concentrations, mixing isotonic (0·6M) $MgSO_4$ with sea water in various ratios. The relation of the time of immersion just required for preventing the extrusion to the molarity of $MgSO_4$ is shown in Fig. 5. Here, too, the effect was reversible. Though sulphate is an anion which has no extrusion-suppressing effect, the possibility of

extrusion being elicited by the $MgSO_4$ solutions themselves has been eliminated, since dilution of sea water with the 0·6 M-$MgSO_4$ solution was always far less than twenty times (see § 1), except for the single plot in Fig. 5 which corresponds to the undiluted, 0·6 M-$MgSO_4$. In fact, there was observed no extrusion in the $MgSO_4$ solutions which were tested, and such was true also in the case of the pure, undiluted 0·6 M-$MgSO_4$. In view of the very short anaesthetization time (3 sec.) which was required for so high concentration as 0·6 M of $MgSO_4$, a Cl-free medium might reasonably be considered to have had no time to be effective before the anaesthesia was established. It is important here to point out that Mg ion suppresses the extrusion much as do certain anions named in the foregoing section, but is different from the latter in that its effect will last for some length of time even after its removal. The term 'anaesthesia' may well be applied to the long-lasting effect of Mg ion in preventing the extrusion reaction, as distinguished from the suppressor action of anions which is effective only during contact. From Fig. 5 it is seen that Mg with concentration less than three times the Mg level of natural sea water suffices to 'anaesthetize' the reaction.

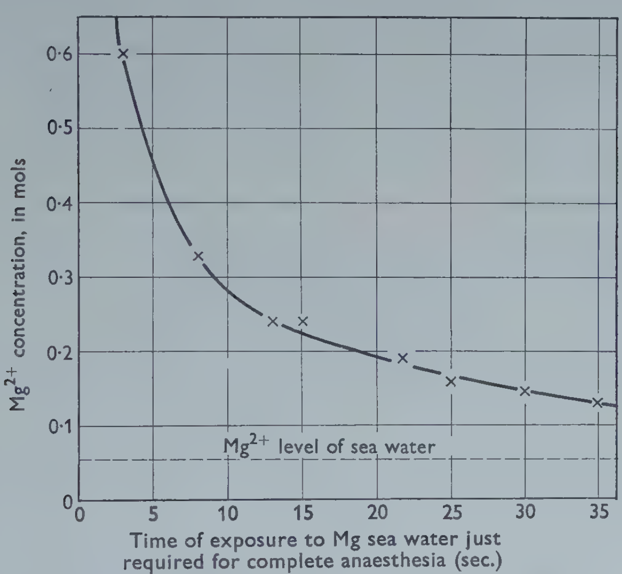

Fig. 5. Strength–duration relation in the anaesthetization of cnida extrusion by $MgSO_4$. The graded concentration of Mg^{2+} was obtained by varying the ratio between 0·6 M-$MgSO_4$ and sea water. Temp. 28–30° C.

It may be added here that on transfer to a Mg salt solution the acontia gave a visual impression that they had been released from some mechanical tension. Thus far, no further inquiry has been attempted into this aspect of Mg effect on acontia, which appeared to relate to the musculature and not to the cnida response.

(*b*) *Organic anaesthetics.* 5 % urethane and 1 % Na-diethylbarbiturate, each in solution in sea water, were found to prevent extrusion completely when the acon-

tium was exposed to 1 M glycerine solution following the anaesthetics. Immersion for 1 min. and for 10 min. were sufficient for urethane and diethylbarbiturate respectively. In both cases the acontium which had once failed to react came to recover its normal responsiveness after being washed free from the glycerine solution with normal sea water and then kept in normal sea water for 15–30 min.

Lipoid solvents like alcohols, ether or chloroform possess generally the effect of bringing about disintegration of the acontial tissue structure, sometimes with accompanying extrusion or discharge of cnidae from the acontium (Yanagita, 1959*d*). In fact, ether was reported as an extrusion-inducer by Parker & van Alstyne (1932) and by Pantin (1942). Such a situation creates a difficulty in testing their possible 'anaesthetizing' effect. For this reason evidence so far obtained of their efficacy is rather scanty, but there are some data which obviously reveal the susceptibility of the extrusion reaction to anaesthetization by the action of lipoid solvents. The following is a representative set of data obtained with ethyl alcohol vapour. In this experiment M/3 Na_2SO_4 solution was used as the extrusion-inducing medium instead of the usual 1 M glycerine, just for a clearer judgement of result (see § 3). Pieces of acontium, stretched out across a small sea-water pool on the slide glass, were exposed to the vapour of ethyl alcohol in small covered dishes for varied lengths of time. Then they were taken out into the open air, the medium was changed to M/3 Na_2SO_4 solution and the effect was noted. The room temperature was 22–23° C.

Time of exposure to the vapour (min.)	Effect of Na_2SO_4
0	Marked extrusion (no explosion)
3	Marked extrusion (no explosion)
4	Marked extrusion (no explosion)
5	Extrusion not marked (no explosion)
6	Slight or no extrusion
6·5	No extrusion, but the tissue soon began to disintegrate

Recovery of reactivity to Na_2SO_4 by acontia which had been anaesthetized by alcohol vapour was confirmed in another set of observations of a similar type, the acontia being washed in fresh sea water for 10 min.

Similar trials were made also with vapour of isopropyl alcohol, ether, chloroform, acetone and of peppermint oil, each giving positive results. Recovery from anaesthesia was demonstrated for these five lipoid solvents, this time by using the control acontia, which were kept long enough in the anaesthetizing medium for anaesthesia to be complete. The times required for complete anaesthesia and for recovery, with the lipoid solvents here dealt with, are given in Table 2. Acetone was already known to have a peculiar action on the acontial surface (Yanagita, 1959*d*), and as to the anaesthetizing effect of its vapour, there is so far only little evidence, owing to a breakdown of the surface structure being readily produced even by a very short exposure to the vapour.

Table 2. *Anaesthetizing effect of lipoid-solvent anaesthetics on cnida extrusion reaction*

Anaesthetics	Time required for anaesthesia (min.)	Time required for recovery (min.)	Temp. (° C.)
Ethyl alcohol, vapour	6	10	23
Isopropyl alcohol, vapour	10	10	14
Ether, vapour	3	10	16
Chloroform, vapour	2	10	17
Acetone, vapour	3	10?	17
Peppermint oil, vapour	20	20	13

DISCUSSION

The nature and mechanism of the extrusion response

As described in the foregoing sections, the capsular bodies of the cnidae, in the extrusion reaction, are shot out of the acontial surface with such briskness as to suggest that the reaction occurs upon some endogenous impulse, and, moreover, the reaction is susceptible to anaesthesia. This makes it likely that the reaction represents activity on the part of the living tissue of acontium, involving an 'excitation' in the proper sense of the word. The same features were noted by previous authors, particularly by Pantin (1942).

Pantin has described the extrusion in the *Anemonia* tentacle as 'a frequent response in irritant solutions'* which was 'always accompanied or perhaps caused by great muscular contraction and secretion of mucus'. The latter statement, however, seems not to hold in the present material. The acontium of *Diadumene* is furnished with a pair of strong tapes of longitudinal muscle fibres running along the middle of its 'ciliated band', and by contraction of these fibres it can be tightly coiled up, wriggled, and so on. Such movements are indeed often found to set in or to be enhanced just at the time when the acontium is placed in an extrusion-inducing medium; but, as already stated in §2, no causal relationship can be found between muscular activity and cnida extrusion. In fact, the extrusion was also observed freely taking place from an acontium which was stretched out with both its ends fixed so as to permit no actual shortening.

Another possibility concerning the driving force for extrusion is protoplasmic contractility, as it is assumed by Abric (1904) for the 'cnidoblast'. However, this remains hypothetical in the present case also since no contractile movement was actually detectable at the acontial surface (see §2). A third, and now hopeful, alternative is a purely physical force due to the 'surface properties' of the cnidae themselves, which has been suggested by Picken (1953, p. 225) as the possible cause of their centrifugal migration through the body tissues and also of their extrusion from the body surface.

* He mentions as such K-rich (M/8–M/4) sea water, as well as ether, triacetin (2 %) and quinine (0·1 %), each dissolved in sea water.

As will be described in a separate note (Yanagita & Wada, 1959), the cnidae in the resting state are embedded in the epithelium of the 'nematocyst band' of the acontium with their distal ends separated from the exterior by a thin (about 2μ in thickness) continuous protoplasmic sheet which, being hyaline and ciliated on the outer surface, can be taken as the cortical layer of the epithelial cytoplasm. Whatever the motive force for the extrusion may be, the cnidae will have first to break through this layer if they are going to be extruded.

The results obtained in the present work concerning the effects of anions in suppressing extrusion suggest that some change in the acontial surface caused by those external ions plays an important role in the reaction in question. A scrutiny of the efficacies of anions in comparison with the lyotropic series may lead to a generalization that such kinds of anions as favour the sol state of a hydrophilic colloid are effective in suppressing extrusion, which would promptly take place in an ion-free medium like a glycerine solution. Such capacity should be absent, on the other hand, from those anions which favour gelation or coagulation, i.e. SO_4^{2-}, tartrate^{2-}, etc., or they might even have the effect of accelerating the extrusion.

It is important here to point out that, if one accepts Picken's concept of a pre-existent propulsive force intrinsic to the cnidae, a simple explanation of the observed facts will be at hand. One must only postulate that gelation of certain (perhaps protein) components of the acontial surface membrane is accompanied in some way or other by the weakening of the mechanical consistency of the surface or even by its partial breakdown. If this were to happen somewhere along the acontial surface the cnidae in that region might at once start to discharge, breaking through the weakened surface and thus giving rise to the phenomenon of extrusion. The additional assumption might reasonably be made that the breakage of the surface is somehow repaired soon afterwards. If, on the contrary, one adheres to a hypothesis based on muscular or protoplasmic contractility as the driving force for extrusion, any discussion of the matter can go no farther than establishment of an empirical law about an 'excitation' of the cell type concerned by one group of anions and an 'inhibition' by the other.

The 'anaesthetic' effects described in §4 must not necessarily be regarded as acting on some excitable system *within* the acontium. The simplest explanation may be that Mg^{2+} as well as organic anaesthetics act directly on the surface membrane of the acontium and alter (in this case, somewhat permanently) its consistency in the same sense as do Cl^- and other univalent anions, i.e. in the sense of stabilization. This is also supported by the very short length of time required for the anaesthetization by Mg^{2+} ions (see Fig. 5).

The significance of the extrusion response in relation to the normal cnida responses

Various agents have already been reported by the writer which evoke a discharge response of the cnidae from the *Diadumene* acontium when acting in sea water, though they did not act directly on the cnidae previously isolated in the external medium. They are: K^+ and NH^+, electric shock (Yanagita, 1959*b*), certain surface-

active agents and lipoid solvents (Yanagita, 1959*c*, *d*), and mechanical contact of various solid 'food' (Yanagita, 1959*b*). It is important here to note that in a 'Cl-poor medium' (i.e. sea water or M/2 NaCl solution diluted with isotonic glycerine so as to contain Cl^- in a concentration sufficient to suppress extrusion, but insufficient to evoke explosion of isolated cnidae), all the agents named above failed to elicit the discharge response from the acontium but usually produced extrusion of unexploded cnidae from the region of acontium upon which they acted (Yanagita, 1959*b*). This indicates that they, too, are extrusion-inducing agents in their essential character, and can exert discharge-inducing action only in association with Cl^- or similar anions in the external medium.

All these circumstances may lead in quite a natural way to a tentative extension of the hypothesis proposed above, as follows. The various stimulants mentioned in the foregoing paragraph should have only an extrusion-inducing capacity, which overcomes the extrusion-suppressing action of Cl^- and other anions (if such were present in the external medium) so as to cause the weakening in consistency of the acontial surface membrane, resulting in cnida extrusion. The cnidae thus extruded would in their turn promptly be caused to explode as soon as the 'stopper' mechanisms situated at their distal tips (Yanagita, 1943) had come in contact with the external anions. Now, as an experimental fact particular to the contact stimulus (with human hair), it is known that the acontium, when placed in a 'Cl-poor medium', will no longer respond to the stimulus with discharge or with extrusion of undischarged cnidae (Yanagita, 1959*b*). This might possibly indicate that in the case of such a natural form of stimulation the change in the acontial surface is represented merely by an increase in ionic permeability, instead of a mechanical weakening or breakdown. If so, the cnidae, as they are still embedded within the acontium, could come in contact with Cl^- ions to cause the explosion whenever the latter have penetrated the thin surface layer of the acontium whose permeability has been increased. In fact, in a normal response of the acontia to a contact stimulus like that of human hair only the stinging threads are shot out of the surface, whilst the capsular bodies are retained inside the surface, often very firmly. Such was not the case with other, more artificial kinds of excitation, where the cnida discharge from the acontia was always accompanied by extrusion of the capsular bodies.

Pantin (1942), working with the tentacle of *Anemonia*, has stated that 'extrusion is not a normal accompaniment of the response to food'. This holds true also for the present material, provided that one uses the term extrusion as he did, i.e. as meaning the liberation of undischarged cnidae. However, according to the present point of view the extrusion, or at least a reaction related to the extrusion such as an increase in acontial permeability, plays an essential role of mediating all the cnida discharge from the *Diadumene* acontium.

Mg^{2+} and other anaesthetics have been known to prevent the discharge responses to the various stimulants mentioned above except the surface-active agents (Yanagita, 1959*c*). This is generally in accord with the results reported by the earlier authors (Parker & van Alstyne, 1932; Pantin, 1942) for different materials. As for the present material, the results might well be explained on the basis of that

'anaesthetization' of the above-mentioned acontial surface reactions including permeability change, which would prevent all the discharge responses of acontium.

Table 3. *A diagram showing the effects of ions (and other kinds of external agencies) upon the* Diadumene *acontium*

(Arrows indicate the relation of dominance in efficacy of one group of agents over an other. Based in part on information from another work of the writer (Yanagita, 1959*b*).

Extrusion-inducers	Extrusion-suppressors
No ions	Cl^-, Br^-, NO_3^-, etc.
(except for Na^+, Li^+, Ca^{2+}, SO_4^{2-}, etc.)	
K^+, NH_4^+	Mg^{2+}
(and non-ionic discharge stimulants)	(and non-ionic anaesthetics)

The results thus far obtained concerning external agencies acting for and against cnida extrusion are summarized in diagrammatic form in Table 3. The hypothesis as extended to include all the forms of cnida response of the *Diadumene* acontium may be summarized as follows. The cnidae as they are embedded in the acontium are considered as being separated from the external medium only by a thin surface sheet of protoplasm which is able sensitively to change its mechanical or permeability properties under the action of ions, electric current, surfactants, etc. All that is necessary for any cnida response to take place is some weakening of consistency or some increase of permeability of that protoplasmic layer, or particularly of its surface membrane. Once this has happened Cl^- ions, which are abundantly present in the external sea-water, come in contact with the distal tips of the cnidae to release the 'stoppers', with the result that the eversion of the stinging threads ensues, at the expense of built-in elastic energy. Such a mechanism might be said to represent the simplest and perhaps most primitive form of excitable system ever known, especially when the cnida's own propulsive force due to its surface properties is admitted. Whereas the excitable systems as we usually know them consist of a trigger mechanism associated with an intracellular system of reactions including katabolic processes, we might have here an extreme instance which involves only the former element, and where the anaesthetization, too, is related simply to the trigger mechanism.

The problem of 'cnidoblast'

The earlier authors, including Parker & van Alstyne (1932) and Pantin (1942), have postulated the existence of a 'cnidoblast' enclosing each cnida which is embedded in the tentacle or acontial tissue of sea-anemones, though they did not demonstrate it morphologically. They assumed that in the responses of the cnidae to external stimulants there intervened an 'excitatory process' which took place in the mother cells, representing each an independent effector (see also Hyman, 1940, p. 389). According to the writer's observations (Yanagita & Wada, 1959), however,

in the full-grown state of *Diadumene* cnidae, a cnidoblast, if such ever exists, is considered to have already been reduced to an indiscernibly thin, membranous coating close to the capsular body, which has migrated into an enlarged vacuole within an epithelial cell of the acontium. It is very unlikely that such a remnant of the cnidoblast would retain a capacity for any act that deserves the name of excitation. If the extrusion and discharge represent a case of 'excitation' at all in the present material, it may be no other than the thin cortical layer of cnida-sheltering epithelial cells which is 'excited'.

Abric (1904), Parker & van Alstyne (1932), and others assume hypothetically an intracellular production of some acid in the course of the excitatory process and attribute to it the role of acting on the organelles to cause their explosion. According to the present results, Cl^- ions in the external sea water, instead of some intracellular agency, should play the role of mediator. They could be brought into contact with the cnidae on which they act, as an immediate consequence of the first step of the 'excitatory' process, which is represented here by the simple surface changes of the acontium, sometimes resulting in the actual extrusion of cnidae.

SUMMARY

1. The acontium of *Diadumene luciae* was found to extrude its nematocysts (microbasic *p*-mastigophores) when it was placed in sea water diluted twenty times or more with distilled water or with some non-electrolyte solution.

2. The effect of dilution of sea water in inducing the cnida extrusion was revealed to be due to the decrease in concentration of Cl^- and Br^- ions which act on the surface of acontium to suppress the extrusion. A similar effect of suppressing the extrusion was also found with many other univalent anions. Most of the plurivalent anions studied lacked such effect.

3. Visible features of the process of cnida extrusion were described, suggestive of a physiologically active reaction.

4. The cnida extrusion could be anaesthetized either with Mg^{2+} ions or with many organic anaesthetics. The anaesthesia was found to be reversible.

5. An explanation of the nature and mechanism of the extrusion reaction was sought on the basis of the present results, and extended to include all the forms of discharge reactions, including the natural nettling response.

The writer wishes to express his sincere thanks to Prof. H. Kinosita of the University of Tokyo for his encouragement during the course of the work and for reading the manuscript. Thanks are also due to the Director and the staff of the Misaki Marine Biological Station for their kindness and for use of the station facilities. The experiments have been carried out under occasional collaboration of Misses T. Wada, M. Inaba, T. Fujita, Y. Shirato, Mrs H. F. Suzuki, and Mr A. Shimadzu, to all of whom the writer feels his grateful acknowledgement to be due.

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THE COMPOSITION OF THE BLOOD OF THE SHORE CRAB, *CARCINUS MOENAS* (PENNANT), IN RELATION TO SEX AND BODY SIZE

III. BLOOD NON-PROTEIN NITROGEN

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(*Received* 14 *March* 1959)

INTRODUCTION

In a previous communication (Gilbert, 1959*a*) it was shown that conductivity values for the blood of *Carcinus* rise with body size until a maximum is reached at a body weight of 35 g. and then steadily fall beyond this weight. Below 35 g. males have a significantly higher conductivity than females. On the other hand, the curve for total osmotic pressure (O.P.) shows no peak at the middle of the range of body size but falls steadily throughout the whole size range, the values for females being consistently lower than for males (Gilbert, 1959*a*). Since it was possible to deduce that the relative proportion of the ions remains constant over the whole size range (Gilbert, 1959*b*), the discrepancy between the ionic composition and the total O.P. must therefore be due to non-electrolyte; the latter should therefore vary inversely with the electrolyte fraction.

The present work reports on the influence of body size and sex on total non-protein nitrogen (N.P.N.).

MATERIALS AND METHODS

Procedures were similar to those already described (Gilbert, 1959*a*). Blood was removed through a cut in the base of the arthrodial membrane at the base of the large chela using a small glass pipette. Anti-clotting agents were not used.

A semi-micro Kjeldahl method was used for the determination of the N.P.N. Essentially the method was that due to Cole (1944) with some modifications (Shaw & Beadle, 1949). Blood was de-proteinized with tungstic acid, centrifuged, and 2 ml. of supernatant digested in a 6 in. $\times \frac{3}{4}$ in. Pyrex boiling tube in a sand bath at $290 \pm 4^\circ$ C. for 5 hr. The mixture was allowed to cool in a desiccator over concentrated H_2SO_4 to exclude atmospheric ammonia, and the following day the ammonia was distilled from it into 0·1 N-H_2SO_4 using a micro-burner and condenser. The back titration was carried out with 0·1 N-NaOH using methyl red/bromcresol green as indicator.

Solutions

Digestion mixture (1). 150 g. solid K_2SO_4 in 200 ml. distilled water. 400 ml. pure conc. H_2SO_4 was added, followed by 34·4 ml. of a saturated $CuSO_4$ solution.

Indicator (2). 0·1 % alcoholic solution of methyl red, and 0·1 % aqueous solution of brom-cresol green in the proportion of 1·3 and 0·7 ml. respectively in 100 ml. of the 0·1 N-H_2SO_4.

Sufficient blood could be removed from each crab for two determinations; the mean of these was used in the calculations.

It was unfortunately not possible to investigate the relationship of body size and sexual maturity thoroughly. Note was taken of the dimensions of about 100 berried females which were among the 400–500 females obtained during the course of this work, and the gonads of a random selection of males were examined for traces of mature sperm. The latter examination was unsatisfactory. Accordingly, the male sexual pleopod was removed as close to the base of the limb as possible and weighed after drying in air; in other decapod Crustacea this appendage undergoes a marked change in growth at the onset of sexual maturity (W. Stephenson, personal communication).

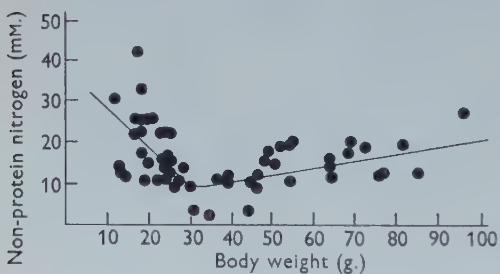

Fig. 1

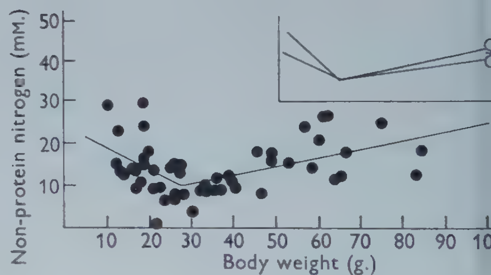

Fig. 2

Fig. 1. Body weight and N.P.N. in male crabs. Linear regression lines were calculated (1) for animals below 35 g. body weight, and (2) for animals above this weight. This procedure was also followed for Fig. 2.

Fig. 2. Body weight and N.P.N. in females. Inset: calculated regression lines for males and females.

RESULTS

Total non-protein nitrogen

The results of the total N.P.N. determinations have been converted to millimoles of nitrogen and plotted against body weight for fifty-nine males in Fig. 1 and fifty-three females in Fig. 2. As expected it can be seen that the curve is roughly the mirror image of that for conductivity, despite the wide scatter of the results. Values for blood N.P.N. fall to a minimum of about 10 mM. for crabs of about 35–40 g. and rise with increasing weight after this region. As in the previous investigation the data for each sex were divided into two groups, above 35 g. and below this weight, and the regression line for each group was calculated by the method of least squares.

The four regression lines, one each for the males and females below 35 g., and one each for the males and females above 35 g., all differ significantly from the horizontal ($P < 0{\cdot}01$ for those below 35 g. and $P < 0{\cdot}05$ for those above 35 g.). An analysis of covariance showed that the regression coefficients for the lines of negative slope do not differ significantly from each other, nor do those of positive slope ($P > 0{\cdot}05$). However, below 35 g. body weight, males have a significantly higher blood N.P.N. than females ($P < 0{\cdot}01$); above 35 g. the N.P.N. of males is significantly lower than that of females ($P < 0{\cdot}01$).

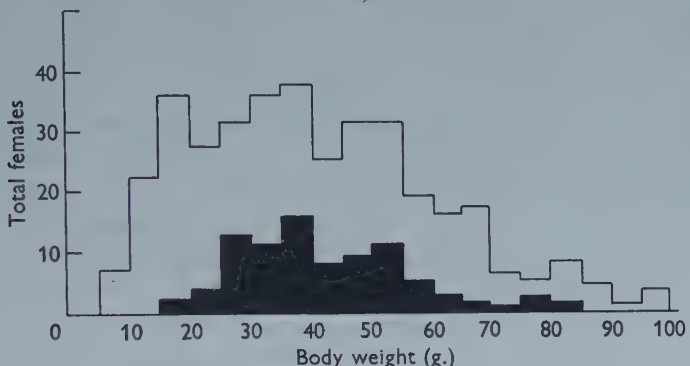

Fig. 3. Frequency distribution of females: 5 g. body-weight class intervals. Berried females, ■.

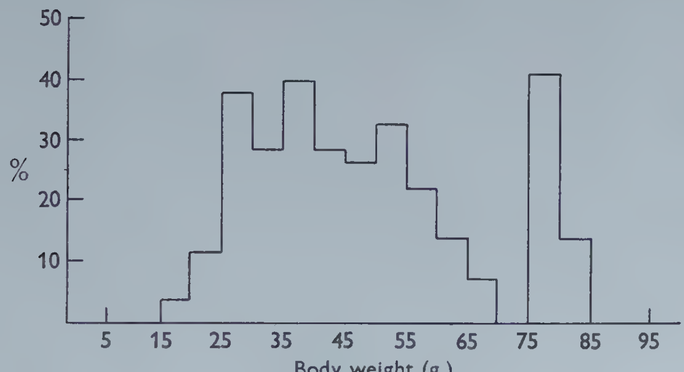

Fig. 4. Berried females as a percentage of total females for each size class. 5 g. body-weight intervals.

SEXUAL MATURITY

The data for the berried females is presented as a histogram (Fig. 3). It is clear that the majority of the crabs in berry are to be found in the region 35–55 g. body weight, less than a third of the size-range containing over 80% of the berried females. However, it is also true that a higher proportion of the animals supplied to this laboratory were in this region of the size range. It is clear that the greatest values are obtained for the region 35–55 g. body weight, even when this is allowed for by expressing the results in percentage units for each size interval (Fig. 4). There is an

apparent exception for the region 75–85 g., but the values for this size are based on three berried females out of thirteen crabs. I understand that berried females of this body weight are extremely rare so that it seems likely that the higher values in this region are due to chance.

The frequency distribution of berried females shown in the figures could be brought about after the initial onset of sexual activity, either by a variation in the frequency of egg production with size, or by sexual maturity being of a finite duration.

Despite the inadequacies of the method of sampling it does appear that the trough of N.P.N. coincides more or less with the most frequent period of reproductive activity in females.

The data for males is even less complete and gives no more than an indication of the onset of sexual maturity. Due to the shortcomings of the method no figures are presented; however, it did seem that males of 25–35 g. body weight had more mature sperm than those of other weights.

In both cases, therefore, the onset of sexual maturity appears to be in the region of 25–35 g. body weight. Orton (1936) reported that the shore crab reaches sexual maturity at a carapace width of 4·5 cm.; this corresponds to a body weight of 23 g. and the results are in reasonable agreement.

The relationship between log body size and log pleopod weight is shown in Fig. 5. It is clear that in the case of *Carcinus* there is no change in the growth rate of the pleopod over the size range taken.

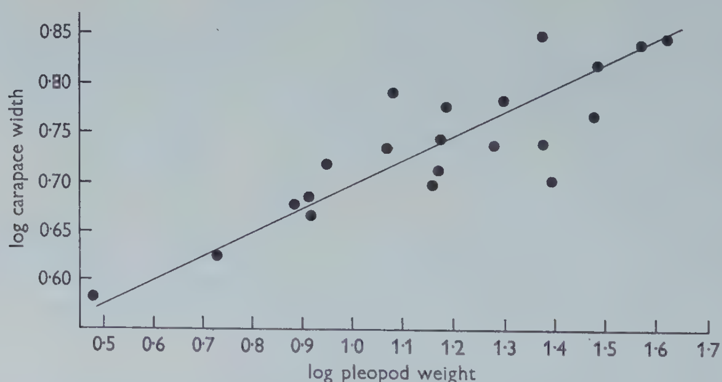

Fig. 5. Log. sexual pleopod weight of males plotted against log carapace width.

DISCUSSION

It has been shown previously that the ionic content of the blood of the shore crab rises with increasing body weight until a maximum is reached at a weight of about 35 g., and after this decreases with further increase in size (Gilbert, 1959*a*). Moreover, since it has also been suggested that the relative proportions of the ions must remain constant over the whole size-range (Gilbert, 1959*b*) it is possible to estimate the total millimolar concentration of the ions at any given weight. By comparison with the results for the freezing-point depression of the blood (Gilbert, 1959*a*) the

millimolar concentration of non-electrolyte can be estimated for any body weight. There is a very close agreement between the calculated figures and those obtained experimentally, except at the two extremes of the size-range. It seems reasonably clear that the concentration of the electrolyte and non-electrolyte in the blood are closely connected. As one fraction increases the other decreases and vice versa, in such a manner that total O.P. remains at a relatively constant level. This suggests that osmotic regulation is important rather than total ionic regulation as postulated by Pantin (1931) and Webb (1940). However, despite the mechanisms for increasing blood electrolyte when the non-electrolyte fraction decreases, the total O.P. is not constant but decreases slightly but steadily with increasing body weight. Moreover, the two sexes differ; females are always hypotonic to the medium and have a significantly lower O.P. than males at any given body weight. At the lower weights males are hypertonic to the medium, at 35 g. they are isotonic, and at higher weights they are hypotonic. Since the control over the electrolyte and non-electrolyte fractions of the blood seems so subtle and the two mechanisms so closely integrated, it seems remarkable that total O.P. is not maintained constant over the whole size-range. It would be of interest to know what function this variation has.

The frequency distribution of the sort shown in Figs. 3 and 4 could be due to a variety of causes, and we do not know enough about the reproductive physiology of this animal to be able to say anything constructive. But from the present point of view it is likely that, apart from berried females, the females examined in the present work would also show the same frequency distribution of reproductive activity as the sexually mature animals. That is, of the crabs of all sizes whose body fluids were examined, it is likely that a higher proportion of crabs of about 35–55 g. body weight would be about to produce eggs or have already done so. It is the crabs of this body weight that gave lowest values of N.P.N. The same reasoning would apply to males. If this view is correct the curves for the variation of electrolyte and N.P.N. concentration with body weight could be due, at least in part, to the different proportions of reproductive animals at any given weight, and in part to the frequency of egg or sperm production; that is, the peak for the mean electrolyte value and the trough for N.P.N. at 35 g. weight might be due to the relatively high proportion of the animals at this weight with high and low values respectively. But clearly only the study of individual crabs through their growth period could show whether this was so.

SUMMARY

1. The influence of sex and body weight on the concentration of the non-protein nitrogen (N.P.N.) in the blood of *Carcinus moenas* was investigated.

2. Blood N.P.N. decreased with body size in both sexes until a minimum was reached at a body weight of about 35 g. Thereafter it increased with increasing body weight.

3. For body weights less than 35 g. males had higher N.P.N. values than females; above this weight male values were lower. Statistically these differences were highly significant.

4. Frequency distribution of reproductive activity with body size showed peaks which correspond with those for total ionic concentration (Gilbert, 1959*a*, *b*) and with the troughs for N.P.N.

5. Results of the present work have been discussed in relation to those reported earlier for conductivity, total O.P., chloride and sulphate (Gilbert, 1959*a*, *b*).

I wish to express my thanks to Prof. A. D. Hobson for his consideration and help at all times, and to express my gratitude to Dr C. Ellenby for his untiring encouragement and advice during the course of this work, and for his criticism of this manuscript. I am also indebted to Mr J. Shaw for many suggestions, and to the staff of the Dove Marine Biological Station, Cullercoats.

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FUNCTIONAL EVIDENCE FOR NEURONE FIELDS REPRESENTING THE INDIVIDUAL ARMS WITHIN THE CENTRAL NERVOUS SYSTEM OF *OCTOPUS*

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(*Received* 11 *March* 1959)

INTRODUCTION

When octopuses were trained after surgical blinding and removal of parts from the central nervous system, individuals were found that could be taught to recognize objects using the arms on one side of the body, but not when obliged to use the arms on the other side; such animals always proved to have lesions extending into the inferior frontal lobe on the non-learning side (Wells, 1959). The training technique used to compare the performance of the two sides was very simple; the same object was presented at short intervals to the same arm of the animal, and the animal was given a weak (6 V. a.c.) electric shock if it grasped the object and passed it to the mouth. An untrained animal will take an unfamiliar object at the first presentation, but if shocked for doing so it learns to reject the object after two or three trials. Animals with certain very large lesions continue to take the test objects despite repeated shocks. When an octopus had shown either (*a*) that it was able to learn (by rejecting the object for a prescribed number of trials, usually six rejections in succession), or (*b*) that it was unable to learn (by making a succession of twenty errors), the object was presented to an arm on the other side and trials were continued. It was found that even animals that learned rapidly on both sides took the object at the first trial after the side-to-side change; and tests with control animals, blinded by section of the peripheral optic nerves but without central brain lesions, showed that the incomplete transfer of the effect of experience was not a consequence of interference with the central nervous system. That one side of the animal has to re-learn what is already 'known' to the other side seemed extraordinary, and the present account is of experiments made to investigate the phenomenon using blind but otherwise unoperated octopuses.

MATERIAL

Octopuses of between 250 and 1000 g. from the Bay of Naples were obtained and kept in aquaria. All of them were blinded by section of the optic nerves and some of them were subjected to a further operation in which parts of the supra-oesophageal brain mass were removed. Details of operational techniques have been given in Wells & Wells (1956, 1957*b*).

In the text, individual animals are identified with a number that consists of a prefix B or E, giving the year in which the particular experiment was made (1955

and 1958 respectively), a serial number, and a suffix indicating the nature of the lesions made. Those that concern us here are B = blind (optic nerves cut between the retina and the optic lobes), NOL = no optic lobes (optic lobes removed by cutting the optic stalks peripheral to the optic glands and sectioning the optic nerves), and NV = vertical lobe removed (two animals only, in the B series). Discrimination experiments reveal no difference in performance between animals blinded by section of the optic nerves and by removal of the optic lobes (Wells & Wells, 1957*b*).

EXPERIMENTAL METHOD

As has been briefly explained in the introduction, animals were trained not to take an object presented to them, by means of weak electric shocks administered under water through electrodes attached to a probe. A 5–10 V. a.c.* shock was given when the animal passed the presented object under the interbrachial web to the mouth; and the object, suspended on a nylon line, was immediately pulled away. The animal was allowed to examine the object until it rejected it, provided that it did not actually take the object under the web. Each trial was thus continued until the animal either passed the object under the web (and got a shock), or let go of it, usually after pushing the object away to the tip of the arm in contact. Provided that an object is small and light so that it can readily be moved, an octopus will normally use only the first arm to make contact in taking it, so that it is relatively easy to ensure that the reaction is carried out by a single arm, at least until the object is passed under the web. The test object was a 2·5 × 3·0 cm. Perspex cylinder (P 1, P4 or P8; details of these objects are given in Wells & Wells, 1957*a*) weighing approximately 5·0 g. in sea water.

Experiments consisted of a succession of negative trials at regular intervals, at each of which the same object was presented to the same arm of the animal under test until the animal learned to reject it, whereupon the test was continued using a different arm. The animals were given no pre-operational experience of the test objects in the early (B series) tests, and each animal was used once only. Subsequently (E series) it was found that animals could be used more than once if they were given positive training to take the object between sets of negative trials, and some of these later tests were made with animals used two or three times.

EXPERIMENTAL RESULTS

A. *Experiments with trials at short intervals*

A 1. *Experiments with trials at 5 min. intervals (B series)*

Six animals were trained using the second arm on the right side until they had rejected the test object five to seven times in succession, whereupon tests were

* Within these limits the voltage of the shock did not appear to make any difference to the performance of the animals in these experiments. Preliminary tests showed that octopuses were less readily trained if shocks < 3 V. were used, and voltages > 10 V. a.c. were avoided because of the possibility of direct damage to the animals.

continued on the left side, using the second left arm. Details of the performance of the right side of these six animals are given in Wells (1959). Two of them had been blinded by section of the optic nerves, and the rest by removal of the optic lobes; in two of the latter the vertical lobe of the brain was also removed—a lesion that does not affect the performance of animals in these experiments (Wells, 1959). Four out of the six octopuses took the test object in the trial immediately following the arm change, and the remaining two took it in the next trial (Table 1A). It is not clear why these two took the object in the second trial after the change, having failed to take it in the first; both, however, rejected the object in the first trial by withdrawing the arm from contact and not by grasping the object and thrusting it away, so that it is quite possible that they failed to grasp it properly on these occasions.

A 2. *Experiments with trials at 3 min. intervals (E series)*

Seventeen series of tests were made with twelve different animals, all blinded by section of the optic nerves. The animals were trained, using the second right or second left arm, until they had rejected the test object six times* in succession, whereupon a change of arm was made as before. In twelve out of the seventeen sets of trials the test object was taken in the trial immediately following the change, and in one at the trial following this; in four cases animals failed to take the test object after a change from side-to-side (Table 1, details of some typical experiments are given in Fig. 1). Most of the animals (all those indicated + in Table 1) were given positive training on the day or days immediately preceding each series of negative tests. This took the form of groups of six trials, three with the second left and three with the second right arm, carried out at a rate of two such groups per day until the octopus took the test object on every occasion throughout a group. At each positive trial the object was presented and the octopus was rewarded with a piece of fish if it took it: if the animal failed to take the object this was presented together with the fish for several trials, after which the animal took the object presented alone. Positive training ensures that the animal will take the (carefully cleaned) test object at the start of a series of negative trials, even though it has previously been subjected to a similar series of negative trials. It also ensures that negative tests with previously untried animals begin with more or less standardized reactions to the test objects.

The results of arm-change experiments are more consistent when animals are first given positive training than when animals are used without pre-training. Of the seventeen series of tests listed in Table 1A, ten were made after positive training and in nine of these the test object was taken in the trial immediately following the arm change. Out of the four animals that failed to take the test object after change of arms only one had positive pre-training.

In four experiments the test object was changed after the second arm to be tested had ceased to take it. The octopuses had not been pre-trained to take this second

* Five times only (by error of the experimenter) in the case of E14B and E59B.

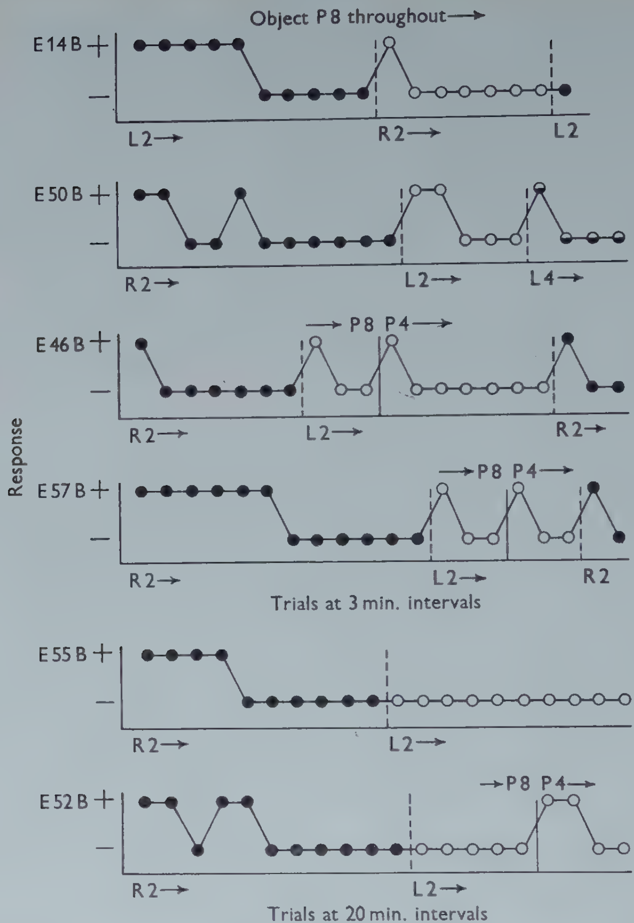

Fig. 1. Results of training animals not to take (+response) an object presented by giving them 5–10 V. a.c. shocks when they did so. The plot of the experiment with E14B shows a typical performance with trials at 3 min. intervals; the animal took the object five times (getting five shocks) when it was presented to the 2nd left arm (L2), rejecting the object (−response) thereafter until this was presented instead to the 2nd right arm (R2). Having re-learned using R2, no further errors were made when a change back to L2 was made. E50B is a similar experiment in which an additional change from the 2nd to the 4th left arm was made, and the object was taken. The experiments with E46B and E57B show that a new object (P4 instead of P1) is taken if presented after the octopus has learned to reject the first, and the arm-change experiment can be repeated. E55B and E52B show the quite different sort of result obtained when trials are at 20 min. intervals instead of 3 min. intervals; the object is not taken following a change of arm but a second object *is* taken if presented.

object, but it was nevertheless taken at once by all four of the animals tested* (Table 1A, and Fig. 1). A further two animals from among those that did *not* take the object following an arm change were tested in the same way, and neither took the new object when this was presented (Table 1).

* A change of arm after the animals had ceased to take the new object resulted in immediate positive responses by three out of these four animals (the performance of two of these, E46B and E57B is given in full in Fig. 1).

The experiments with trials at 3 and 5 min. intervals may be summarized as showing that a side-to-side change of the arm tested almost always causes the animal to take an object presented when it would not otherwise do so. Evidently the experience of one arm is not completely available to the arms on the other side of the same animal. This is curious because we know that learning not to take an object is a function of the central nervous system and not of the individual arms since animals with the supra-oesophageal lobes removed will take the same object ten or twenty times in succession and receive as many shocks, without apparent change in their reactions to the object (Wells, 1959). This being so, the experiments quoted must indicate some functional subdivision of the nerve cells and neuropil comprising the tactile learning areas; changes occurring in the nerve field directly related to one arm do not necessarily determine the reactions made by the rest.

Training the first side of the animal does, however, affect the result of training the second side since the number of errors made by the second arm is rarely as large as that made by the first. In the nineteen trial series listed in Table 1 animals averaged 4·1 errors for the first arm to be tested, and 1·5 errors after the change of sides. This could be interpreted as showing a decremental spread to the other side of the animal of the effect of training to recognize the specific object. An alternative explanation is that there is an unspecific effect of a succession of shocks depressing the probability of positive responses to all objects by all arms to some extent (see p. 507).

A 3. *Experiments with trials at short intervals in which the arm change was between arms on the same side of the animal*

The training technique used for these experiments was the same as that used for testing side-to-side changes with trials at 3 min. intervals. Generally the tests were made on animals that had already been subjected to a side-to-side arm change in the same set of trials (one such, E50B, is included in Fig. 1). Six animals were used in these tests. With three of them the object was presented to an arm next to one already trained, and in one of these three cases the object was taken in the trial immediately following the change. The other three were tested by changes between arms on the same side separated by an intermediate arm (e.g. L2 to L4); two of these three took the object in the trial immediately following the change.

In considering these results it must be remembered that although an octopus takes an object and passes it under the web towards the mouth using one arm only, the bases of the arms near to that actually making the reaction inevitably take part in the later stages of moving the object in towards the mouth and are liable to receive a shock. The shock is given by touching the web over the base of the arm that has bent under to pass the object to the mouth; the chromatophores in the web in this area go momentarily very dark as the shock is given, and if the extent of this darkened area is a reliable indication of the region immediately excited by the shock (as seems probable), there can be no doubt that the bases of adjacent arms are shocked simultaneously with the base of that initially concerned with the object. It is therefore particularly difficult to assess the results of changes between adjacent arms,

and to some extent this difficulty applies to any arms on the same side of the animal, because we have no means of rating the experience of the second arm before the change. Any experience acquired by the second arm before the change would, however, tend to reduce the probability of its reacting positively, so that the results of these experiments (object taken three out of six times) strongly suggest that arms on the same side of the animal, like arms on opposite sides, do not share a common pool of experience.

Table 1. *A summary of results of experiments in which octopuses were subjected to a succession of negative trials*

(Each entry gives the result of a test in which an object was presented to an arm of an octopus that had already been trained to reject the same object using an arm on the opposite side of the body.)

Object was taken in the trial immediately following the side-to-side change	Object was *not* taken in the trial immediately following the change	
	Object taken at the 2nd or 3rd trial after the change	Object not taken at all after the change
A. Experiments with trials at short intervals		
B series (5 min. intervals)		
B 120 NOL	B 121 B (2nd)	Nil
B 127 NOL	B 123 B (2nd)	
B 112 NVNOL		
B 113 NVNOL		
E series (3 min. intervals)		
E 14 B	E 23 B (2nd)	E 39 B –
E 38 B		+E 40 B –
+E 48 B		E 21 B
+E 50 B		E 21 B 2
E 50 B 2		
+E 46 B 2* Twice		
+E 53 B 2		
+E 55 B 2		
+E 53 B 3*		
+E 55 B 3		
+E 57 B* Twice		
+E 56 B 3* Twice		
Total tests giving this result 16 (19 including those tested twice in the same set of tests, see Fig. 1)	3	4
B. Experiments with trials at relatively long intervals		
E series (20 min. intervals)		
+E 59 B	+E 53 B	+E 46 B
+E 46 B 3	+E 56 B 2 (3rd)*	+E 52 B*
	+E 60 B 2 (2nd)*	+E 55 B
	+E 63 B (3rd) –	+E 56 B
		+E 56 B 4
		+E 60 B
Total tests giving this result 2	4	6

+ = Animals had positive training to take the object before this test.
* = The test object was changed, after the arm change, and taken.
– = The test object was changed, after the arm change, and *not* taken.

Figures 2, 3, etc. (not in parentheses) indicate animals being used in a second or third series of tests; in most cases animals were given positive training between successive sets of negative tests. Several of the animals were used in both long and short interval tests.

'Twice' means that the result was repeated after a change of object (see Fig. 1). E 53 B 2 was also tested in this way but failed to take the object on the 2nd change.

B. *Experiments with trials at relatively long intervals*

Quite different results were obtained in experiments with trials at intervals of 20 or 30 min. When training was carried out, exactly as before but with trials 20 min. apart (in one case 30 min. apart) instead of 3 min. apart, octopuses learned to recognize and reject the test object after about the same number of errors as before (mean 4·6 errors with trials at 20 min. and 4·9* with trials at 3 min.), but differed in that they did *not* start to take the object again when a change of arm was made in the middle of an experiment. The test object was taken in the trial following a side-to-side change of arm in only two out of the twelve sets of tests made under these conditions with eight different animals. Six of these eight animals were also used in experiments with trials at 3 min. intervals, and in these all six took the test object when the arm was changed (Table 1). The test object was itself changed in five of the experiments with trials at long intervals, and in four of these it was taken at once; the lack of response when a change of arm was made with the first object was not, therefore, due to an unspecific cessation of positive responses. These results are in keeping with an observation made elsewhere (Wells & Wells, 1957*b*) that selection of a particular arm for training is unimportant in discrimination training when trials are at long intervals.

DISCUSSION

Experience specifically associated with the test object is not, of course, the only factor that may influence the reaction of an octopus to that object. The time since operation, the general condition of the animal and the interval since the last shock or meal are all liable to affect the probability of a positive response. Factors of this sort might conceivably contribute to the difference in results of experiments with trials at long and short intervals, and before considering the implications of this difference this possibility must be examined.

The factors known to affect the probability of a positive response are:

(1) *The time since operation.* Immediately after operation blind animals refuse food and reject all objects presented to them. This phase is transient and within a few hours they begin to take food and thereafter over the next few days become steadily more likely to take objects touched (Wells & Wells, 1956). This could affect the result of training experiments if these were made within a day or so of the operation, because there would then be a significant rise in probability of positive response over the period of the experiment, particularly if the trials were spread over a considerable period. In the present series of tests, however, animals were not used until they had fully recovered from the operation and, indeed, it is a prerequisite of the training technique that animals at once take unfamiliar objects presented to them.

* As all the animals subjected to a succession of negative trials at 20 min. intervals were first given positive training, this figure is calculated from the nine cases in which positive training was given before negative trials at 3 min. intervals.

(2) *The general condition of the animal.* Sick octopuses and female octopuses with ripe gonads (Wells & Wells, 1959) do not take objects presented to them, and reject food. As a check on the condition of the animals these were always given food immediately after the last negative trial in each set of tests and results obtained with any that failed to feed were discarded.

(3) *The time since the animal was last fed.* Young (1958) has shown that in visual training experiments feeding the animals increases the probability of positive responses. After food the probability of an attack on any figure shown rises, and declines over the next 2–3 hr. This could affect the results of experiments if the animals were fed within 2 or 3 hr. of the first trial, and in the bulk of the experiments quoted in this account (all of the E series) animals were fed not less than 12 hr. before test.

(4) *The time since the last shock.* If a blind octopus is given a succession of small electric shocks it reverts for a time to behaviour resembling that found immediately after operation. It will snatch away its arms if touched and refuse to take objects presented to it. The animal will again take objects presented to it only after a period that depends upon the number and strength of the shocks given; the effect of a single shock of the strength used in the present experiments will last a few minutes, that of ten or twenty such shocks for several hours (Wells & Wells, 1956). This unspecific effect of electric shocks occurs regardless of whether the shocks are given in association with a particular object or not. Quite apart from its effect in producing relatively long-lasting changes in response towards a specific object, a shock given in training reduces the probability of a positive response at the next trial whatever the object presented. This becomes less marked at each succeeding trial.

(5) *The spontaneous drift towards positive responses.* A healthy blind octopus that has fully recovered from the immediate effects of operation will take and try to eat any small movable object that it encounters. When the animals are trained to discriminate between two such objects by far the greater fraction of the errors that they make is due to acceptance of the 'negative' object that they are shocked for taking; errors due to non-acceptance of the 'positive' object, which they are rewarded for taking, are comparatively rare (Wells & Wells, 1956, 1957*a*). When training ceases the animals slowly revert to indiscriminate taking of both objects and in retention tests nearly all mistakes are due to acceptance of 'negative' objects (Wells & Wells, 1958). This constant drift towards positive responses would appear to be why blind octopuses rarely make 100% correct responses for any length of time in discrimination experiments even after long training and it can be shown that even when training is limited to a succession of negative trials (as in the experiments quoted in the present report) the performance of octopuses never becomes perfect if trials are spread out over a period of days (Fig. 2). In the present series the effect of a drift towards positive responses is that the probability of the animal taking the test object rises slightly with each successive trial during any series in which the octopus does not make a mistake.

It will be observed that this last factor, like the effect of shocks and the time elapsed since operation, would operate to increase the probability of positive re-

sponses with the passage of time so that the increase per trial would be greater in those experiments with a relatively long interval between trials. But this is not the result observed. A change of arm after six successive negative responses is much more likely to produce a positive response when trials are 3 min. apart than when they occur at 20 min. intervals. Clearly these factors would act against the results observed, and cannot be responsible for them. The evident spread of the effect of experience with time is therefore a feature of the tactile learning process in octopus and not an experimental artifact. It implies that: (1) The inferior frontal/subfrontal lobe system, that has been shown to be the site of tactile learning in octopus (Wells, 1959) must be functionally subdivided at least into two halves representing the two sides of the animal and probably into fields corresponding to the individual arms, observations that fit with what we already know about the organization of the octopus's tactile system (see below). (2) Events affecting the neurones of one field do eventually affect those of other fields, but this takes time, the full effect not becoming apparent for several hours.

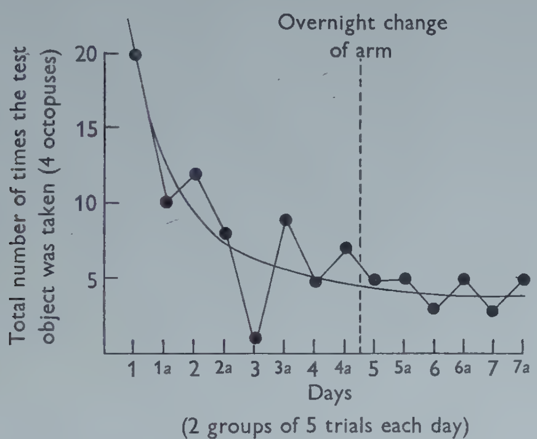

Fig. 2. Results of an experiment in which a large number of negative trials was spread out over a period of 7 days. The individual trials were in groups of five, at 5 min. intervals; on each day there were two such groups—the second group is indicated 1 *a*, 2 *a*, etc. It can be seen that improvement in performance after the first 2 days is very slow because animals tend to revert to positive responses between groups of trials, and it seems likely that under these conditions performance would never become perfect, however long training was continued. A change of arm after 4 days showed that by this time the effect of experience of one side had spread across to the other.

(1) is to some extent borne out by the gross structure of the parts concerned. The subfrontal lobe (but not the median inferior frontal) is obviously divided into two lateral halves, though there are extensive connexions between the two. There is no such obvious division into arm fields. (2) is interesting because of the order of the time interval that must apparently elapse before the effect of events directly concerning one functional area begins to determine the actions of the rest. This time interval is of the order of hours. This is a curiously long time if the spread of changes determining negative instead of positive responses in a given situation is

entirely attributable to electrical events. It is, on the other hand, a reasonable period if these changes are a result of growth processes affecting the connectivity of the neurones concerned. This suggests that we are dealing with processes at first electrical that become entrenched structurally with the passage of time, by no means a new idea (see, for example, Young, 1951) but shown here perhaps more convincingly than usual.

The observation that the neurone fields of the individual arms are to some extent functionally independent is consistent with what we already know about the organization of the tactile system of the octopus. The motor responses to objects touched are few and relatively stereotyped; they can be demonstrated in isolated arms and must therefore be integrated in detail within the axial nerves of the arms concerned, the function of the brain being limited to selection and initiation of one or another of a small repertoire of essentially chain-reflex responses (Wells & Wells, 1957*a*). There is no evidence from the way that octopuses, with and without brain lesions, handle objects to suggest that use of more than one arm at a time involves central integration of the activities of the individual arms, any co-operation that occurs being explicable as a direct consequence of their radial arrangement around the mouth (Wells, 1959). Rapid pooling of the sensory inputs from the individual arms is not therefore a prerequisite of efficient acceptance or rejection of objects touched. This is not, of course, true of postural adjustments made necessary by such movements. For these, as in the movements of walking and swimming, rapid pooling must take place. The parts of the brain dealing with touch learning can, however, be shown to be structurally and functionally separated from those dealing with proprioceptive information (apparently *not* available as a basis for learning), and this departmentalization is seen to be less surprising when the habits of cephalopods in general and the probable phylogeny of *Octopus* are taken into account (Wells, 1959).

The limitations of a system in which the right arm does not know what the left has just done would appear at first sight to be biologically disastrous, but on closer examination is seen to present no special problems in an animal where the information is not required for integration of motor activities. Situations in which an important succession of events affecting one arm only is rapidly followed by similar stimulation of another arm (as in the situation created in the experiments) must be rare in the sea. The possibility is clearly not important when the animal can see, but it could, in principle, become important when the creature is groping under rocks for crabs or other prey. It is conceivable that an octopus encountering, for example, an unusually large and resourceful crab would sustain serious injury to the first arm to make contact, but a delay in the distribution of information about this event to the other arms is hardly likely to increase their chances of mutilation. When an octopus is badly hurt, in whatever part of the body, it moves rapidly away, and the probability of further injury is not governed by the evident limitations of its tactile learning system. It will be noticed that the artificial conditions of the present training experiments did not allow this possibility as a means of avoiding repetition of painful situations, although receipt of an electric shock was nearly

always followed by the octopus's retreat to another part of the aquarium; in the sea it is highly probable that retreat always avoids involvement in situations where the limitations of the tactile system might threaten survival.

SUMMARY

1. Octopuses blinded by section of the optic nerves were trained by means of 5–10 V. a.c. shocks to reject objects that they would otherwise take.

2. With trials at 3, 5, or 20 min. intervals, in which the test object was always presented to the same arm, animals learned within four or five trials, thereafter rejecting the test object whenever it was presented.

3. When, after a succession of such negative responses, the object was presented to another arm on the other side of the octopus, the result depended upon the rate of training before the change. Thus the object was taken in the trial immediately following the arm change in nineteen out of twenty-six sets of tests with trials at 3 or 5 min. intervals, but in only two out of twelve sets with trials at 20 min. intervals; further experiments in which changes were made between arms on the same side produced similar results.

4. These results are interpreted as showing that changes occurring as a result of experience directly affecting one arm take a period of several hours to spread and become effective in determining the reactions of the rest. This in turn implies the existence of functionally independent neurone fields representing the individual arms, and is discussed in relation to what is already known about the organization of the tactile system of the octopus.

The author would like to thank the director and staff of the Stazione Zoologica, Naples, for their hospitality while these experiments were being made, and Prof. J. Z. Young, F.R.S., and Mr B. B. Boycott for their helpful criticisms of this account in preparation.

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VISUAL SCANNING IN THE DESERT LOCUST *SCHISTOCERCA GREGARIA* FORSKÅL

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(*Received* 2 *April* 1959)

INTRODUCTION

Locust nymphs while sitting sometimes sway the anterior part of the body from side to side. The movement was first commented on by Kennedy (1945), who observed it in the field and coined the term 'peering' to describe it, believing that it was correlated with vision. Ellis (1953) agreed with this. Chapman (1955), however, regarded its significance as 'doubtful' since he had observed that it sometimes occurred in a uniform visual field.

Peering is frequently seen when locust nymphs are in the process of orientating to objects in the visual field (i.e. turning towards them (Wallace, 1958)). This strongly suggested that it was related to vision, and a laboratory investigation was made along these lines. The present paper is an account of this work and falls into two main parts: (1) A study of the sort of peering movement observed and its relation to the position of objects in the visual field. (2) A study of peering in relation to the visual estimation of distance.

SIZE OF PEERING ANGLE AND THE POSITION OF OBJECTS IN THE VISUAL FIELD

This experiment was designed to test whether or not peering was influenced by the position of an object in the visual field.

METHODS AND MATERIALS

The swaying of the anterior part of the body is accomplished by straightening the first and second legs on one side and bending the corresponding members on the opposite side. The hind legs are seldom involved and none of the six feet is moved. By stretching the left legs, for example, and bending the right legs the body is made to sway to the right. During the movement the head and body are not tilted, the dorso-ventral axis of the head remaining vertical. Thus in peering the insect moves the whole body except the posterior tip of the abdomen, which rests on the ground so that, in swaying, the longitudinal axis of the body moves roughly like the radius of a circle with its centre at the tip of the abdomen. In some cases the head moves with respect to the long axis of the body (see later, p. 523).

In view of the nature of the peering movement it was decided that this could best be measured as the angle between the various positions of the longitudinal body

axis taking the position of this axis before the peering as a 0° reference line and measuring peering angles to right and left of this. The instrument used was a protractor with a movable pointer. The whole was made of Perspex, the pointer being a broad sector of Perspex with a fine line etched on it. The breadth of the sector allowed the insect to be viewed through it without distortion.

During the experiments the instrument was held horizontally, approximately 1 ft. above the insect and behind it, in which position it did not disturb the insect. It could not be held directly above since with the lighting overhead this would have cast a shadow on the animal.

The longitudinal axis of the body was aligned with the 0° line on the protractor scale, the centre of the protractor being visually superimposed on the tip of the abdomen. When the insect's body moved the pointer was moved by hand to follow it, keeping the line of the pointer on the longitudinal axis of the body. The peering angle was read off in degrees.

With the instrument held as described above, trial measurements made of unknown angles drawn on paper showed that accuracy could be obtained to within one degree. The accuracy achieved in the experiments was probably less since the insect was moving, albeit very slowly.

It was found that a locust nymph placed on a narrow platform would walk along to the end of it, stop and peer. This method was therefore used to position the insects at a known distance and direction from an object in the visual field. The platform was placed inside a white-walled, white-floored arena 2 ft. in diameter and with a wall 10 in. high. Lighting was from overhead. The object was a black stripe 10 in. tall and 2 in. broad stuck on the arena wall. Nymphs of the desert locust are attracted to such objects (Wallace, 1958).

The long axis of the platform was taken as the line of bearing 0° since almost all insects settled with their bodies initially on this line.* In the statement of results the bearing of the object in degrees is therefore expressed with reference to this line.

The insects used were fifth-stage nymphs of the phase gregaria (Uvarov, 1928) since their large size facilitated measurement of the peering angles.

The experiments were performed in a constant-temperature room maintained at 28° C.

EXPERIMENT

Two groups of tests were made, one with an object placed in front of the animals and one with an object at the side.

Object in front. In this test the object was placed directly in front of the insect at a distance of 6 in. At this distance it subtended a horizontal angle of 20° and thus when placed symmetrically on the 0° line its vertical edges lay 10° to the right and left respectively (Fig. 1*a*).

Object at the side. In this test the object was at the right-hand side again at a distance of 6 in. The bearings of its vertical edges were 40° and 60° respectively (Fig. 1*b*).

* Insects which did not take up this position are excluded from the results.

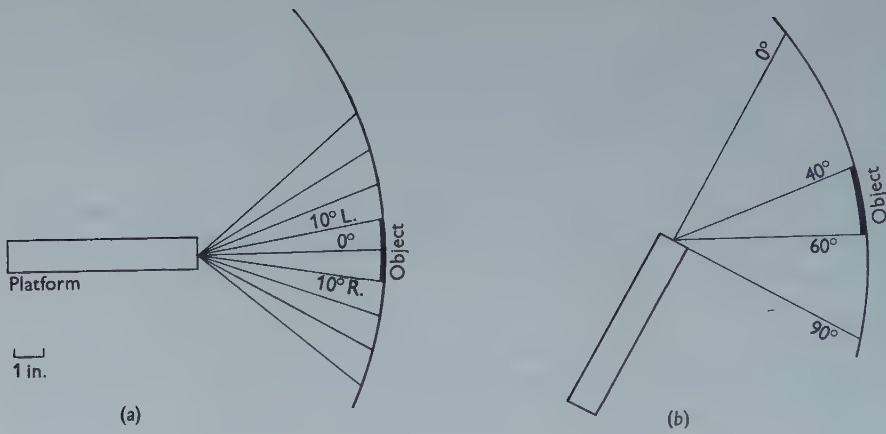

Fig. 1. Plan view showing relative positions of platform and object in an experiment to study the effect of object position in relation to the peering observed. (*a*) Object in front. (*b*) Object 40–60° to the right.

RESULTS

The distributions of the different-sized peering angles in the two situations are presented in Fig. 2*a*, *b* and *c*.

The angles of peering varied from 1 *to* 12°. For convenience they are divided into four categories, 1–3°, 4–6°, 7–9° and 10–12°. In Fig. 2*a* the total frequency of occurrence of angles in each group is plotted irrespective of whether they are to right or left. This is done for both situations. In Fig. 2*b* the frequency of occurrence of different-sized angles to right and left is plotted for the situation where the object is in front. In Fig. 2*c* similar values are plotted for the situation with the object at the side.

From Fig. 2*a* the following points can be made. The maximum angle recorded is 12°. In both situations angles of peering between 4 and 6° are most common. When the object is in front there is a high proportion of angles of 4–6° and angles of 7–12° are rare. When, however, the object is at the side the distribution is different. Angles of 4–6° are still most common but not significantly more so than those of 7–9°. The frequency of angles of 7–9° is significantly higher when the object is at the side than when it is in front ($P < 0{\cdot}01$ by χ^2).

The two remaining histograms show the frequency with which certain sizes of peering angle lie to right or left of the mid-line. It is at once clear (Fig. 2*b*) that with the object in front the distribution to the right is the same as to the left. The distribution is different when the object is at 40–60° to the right. The smaller angles of 1–3° and 4–6° are still distributed equally to right and left, there being no significant difference between the corresponding points ($0{\cdot}1 > P > 0{\cdot}05$, $P > 0{\cdot}99$ respectively by χ^2). The larger angles 7–9° and 10–12° show a marked difference in distribution, there being a significantly greater number to the right, i.e. to the same side as the object ($0{\cdot}05 > P > 0{\cdot}02$; $0{\cdot}02 > P > 0{\cdot}01$ respectively by χ^2).

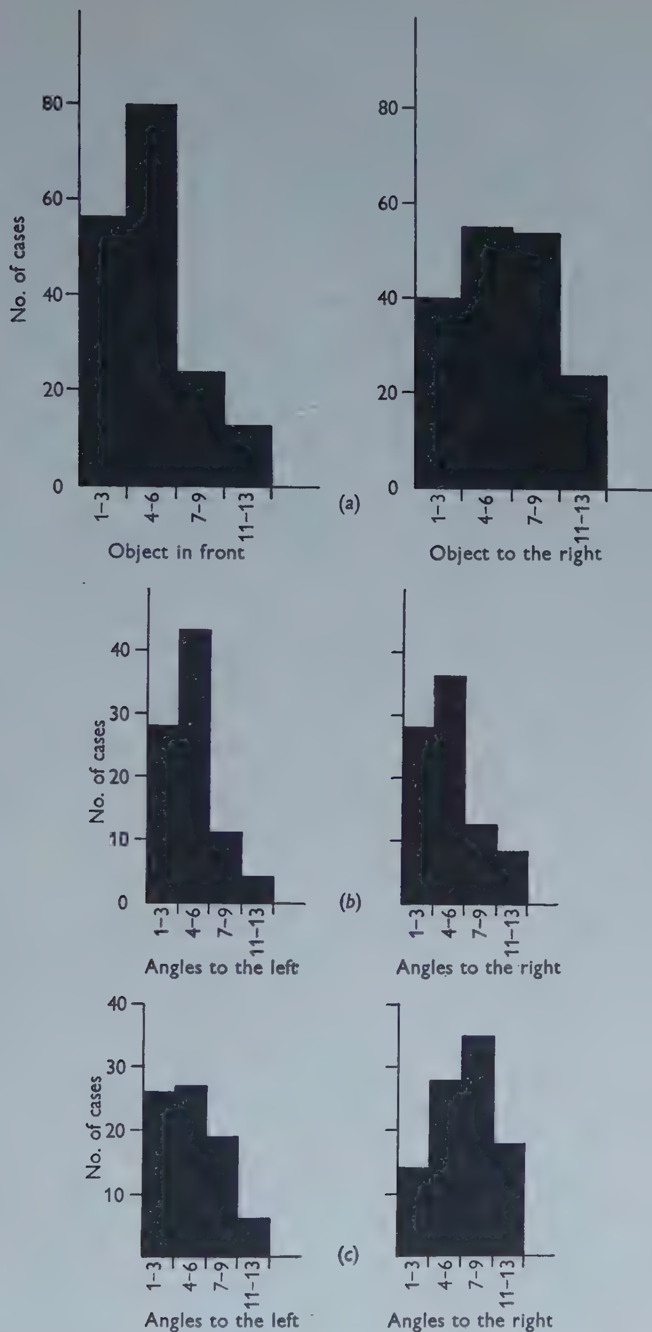

Fig. 2 (*a–c*) Histograms representing the various distributions of peering angles in the two situations shown in Fig. 1. The *x*-axis represents four categories of peering angle: 1–3°, 4–6°, 7–9° and 11–13°. The frequencies of occurrence of these categories are plotted on the *y*-axis as number of cases. The total number of angles recorded for the two situations (object in front, object at the side) is 173 in each case. (*a*) This shows the distribution of peering angles in both situations irrespective of whether the angles are to the right or left. (*b*) This shows the distribution of peering angles to right and left when the object is in front. (*c*) This shows the distribution of peering angles to right and left when the object is to the right.

DISCUSSION

The complete results may be stated as follows. The frequency of small peering angles is not affected by the position of an object in the visual field. When an object lies in front of the animal most peering angles are small and all angles occur equally to right and left. When, however, the object is at the side, there is a significant increase in the number of large peering angles and most of these lie to the same side as the object. The smaller angles are still equally represented to right and left.

Since, then, the position of an object in the visual field influences peering in this way, it can be concluded that the movement is correlated with the visual sense. The fact that the distribution of the smaller angles is unaffected does not of course mean that these are not correlated with vision. As will be mentioned later it is possible that this is a preliminary scanning.

Peering and distance estimation

The above experiment had shown that peering was related to vision but had provided no information as to the function of the movement. Both Kennedy (1945) and Ellis (1953) suggest that it 'sharpens' vision, presumably in the sense of increasing visual acuity. This hypothesis would be difficult to verify.

There was, however, a second possibility, namely, that it was a means of estimating distance. Observation had shown that the nymphs often jumped accurately on to objects and that in all cases (except when the insects were frightened) such jumping was preceded by peering. This ability to jump on to objects was therefore studied with respect to the peering observed.

FIRST EXPERIMENTAL SERIES

This series was designed to test whether or not nymphs discriminated between objects at different distances, to study the importance of certain distance cues and to observe the occurrence of peering in the behavioural sequence preceding jumping.

Apparatus

The narrow platform already described was placed inside the arena with overhead lighting. The long axis of the platform was taken as the 0° line and corresponding reference lines were drawn on the floor at intervals of 5° from 0° to 90° to right and left. The direction of the insect's body could be noted by visually aligning the longitudinal body axis with one of the floor lines. A plain white barrier 2·5 in. high was placed right across the arena between the platform and the objects. The platform itself was 2 in. high and therefore when an insect was sitting at the end of it the barrier cut off all lines of sight below the horizontal, thus preventing any estimation of distance using the positions of the objects' bases.

Since fourth-instar nymphs jump more readily than fifth instars and since they have a sufficiently large body size to make observation easy, nymphs of this instar were used in all the remaining tests.

Behaviour

When placed on the platform an insect would usually walk along to the end of it, stop, peer, then after a pause change the body position and peer again. Changes of orientation always accompanied by peering might occur several times. Finally the insect would usually jump on to one of the two objects present.

Readings

The body position at every orientation was noted and the final choice of object recorded. The actual peering angles were not noted. Each insect was tested five times. A summary of the test situations and the results is given in Table 1.

Table 1. *Summarized conditions and results of jumping tests with two stationary objects*

(The columns N and D show the number of jumps made on to the near and distant objects respectively. Numbers in parentheses represent jumps not aimed at either object. The objects were black rectangles of the dimensions stated.)

Test	Experimental conditions		Choice		Total	P by χ^2
	Near object	Distant object	N	D		
1	6×1 at 3 in. and 45° L	12×2 at 6 in. and 45° R	23	2	25	<0·01
2	4×1 at 3 in. and 45° R	12×2 at 6 in. and 45° L	23	2	25	<0·01
3*a*, partially blinded	6×1 at 3 in. and 45° L	12×2 at 6 in. and 45° R	14	1	15	<0·01
3*b*	6×1 at 3 in. and 45° L	12×2 at 6 in. and 45° R	20 (3)	2	25	<0·01
4, partially blinded	4×1 at 3 in. and 45° L	8×2 at 6 in. and 45° R	14 (1)	5	20	0·05–0·02
5, one eye blind completely	4×1 at 3 in. and 80° R	8×2 at 6 in. and 30° L	12 (1)	2	15	<0·01
6*a*, partially blinded	4×1 at 3 in. and 80° R	8×2 at 6 in. and 30° L	15	5	20	0·05–0·02
6*b*	4×1 at 3 in. and 30° L	8×2 at 6 in. and 80° R	19	1	20	<0·01

Test 1

The purpose of this test was to see if the insect could distinguish between two objects at different distances but subtending the same vertical and horizontal angles from the insect's position (so that their retinal images were the same size) and with their images appearing on corresponding parts of the two eyes.

The objects were two black rectangles. One was 6 in. tall and 1 in. broad at a distance of 3 in. from the insect and at a bearing of 45° to the left. The other was 12 in. tall and 2 in. broad and at a distance of 6 in. from the insect and 45° to the right.

Test 2

This was similar to test 1 except that the nearer object was reduced to a height of 4 in. and placed at 45° to the right. The distant object was now on the left. By thus reversing the positions of the objects, this tested the probability of the insects orientating to some positional cue in the environment. Reducing the size of the nearer object ensured that the image of the further object was always slightly larger than that of the nearer one, irrespective of the position of the insect on the runway.

Tests 3–6

These tests were to test the importance of binocular vision in the estimation of distance. The insects were blinded wholly or partly in one eye. Since locusts are photopositive they might tend to turn to the unblinded side, and for this reason in the first few tests, where the objects were on either side, the nearer object was placed on the blinded side. If there were any bias due to blinding it would be towards the side of the distant object.

Under a dissecting microscope the insects were blinded by application of three coats of cellulose acetate paint. They were examined at the beginning and end of each experiment and in any case where the blinding was faulty as seen by holes or thin places in the cover the insect was rejected. In a preliminary test the insects were tested 3 hr. after blinding but this did not appear to be a sufficient length of time to allow recovery. They showed little inclination to jump. They walked to the end of the platform and then walked all round the end, down the side, often turning several times and moving sideways over the edge. In some cases the animals which jumped somersaulted and missed the object, or jumped, banged into the object and dropped. After 24 hr. they jumped more readily. In all cases therefore the insects were kept for 1 day before testing.

The extent of blinding was checked by observation of the pseudopupil. This is seen apparently on the surface of the compound eye when the eye is viewed under incident illumination. It arises from a group of ommatidia whose optical axes are parallel, or nearly so, to the line of sight. The dark effect is due to total absorption of the incident light by the pigment lying around the retinular cells. The centre of the pseudopupil thus represents a line of sight which is directed along the optical axis of the underlying ommatidium (after Burtt & Catton, 1954). Binocular vision in insects arises as a result of the intersection of the lines of sight of ommatidia in the two eyes. It is therefore clear that binocular vision exists at any point of the visual field from which the pseudopupils of both eyes can be seen simultaneously. The blinded insects were therefore scrutinized under a low-power dissecting microscope and the regions of binocular vision which remained after blinding were ascertained by the above method. An attempt was made to allow for the fact that the ommatidial visual field is greater than that subtended by the ommatidial angle (Burtt & Catton, 1954). This was done by painting out more than the minimum region indicated as necessary from observation of the pseudopupil.

Details of the blinding in each case and the positions of the objects are given in Fig. 3.

In all the tests the aim was to remove the field of binocular vision with the minimum upset to the rest of the visual field. When positive results were obtained in the initial tests, the extent of blinding was increased in the later ones (with both objects on one side) in an attempt to preclude even the remotest possibility of binocular vision.

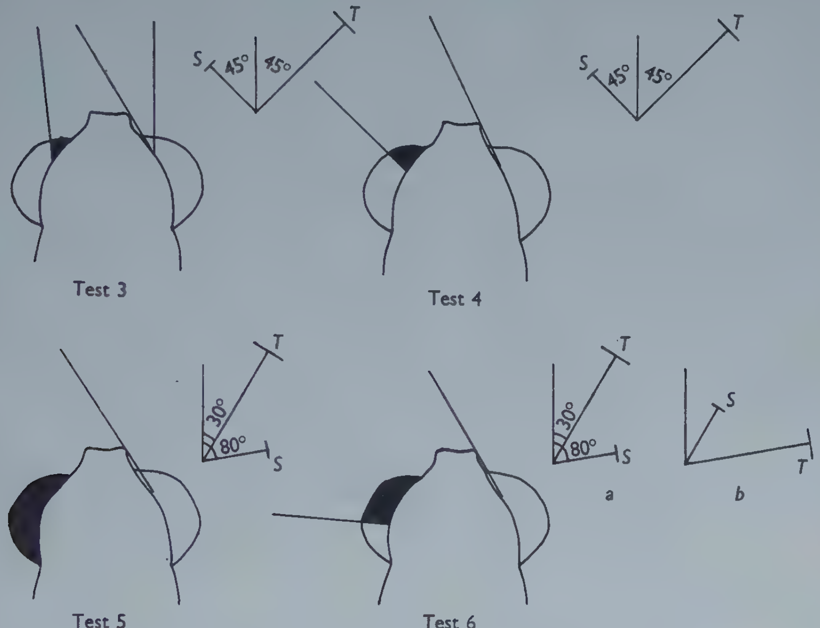

Fig. 3. Diagram showing the area of the eye blinded and fractions of the object in the jumping tests. The head and eye are seen in plan view. For method of estimating the extent of blinding see text. The angles are given with respect to the long axis of the platform (see text). S = short object, T = tall object. Details of object size are given in Table 1.

Results of the first experimental series (*tests* 1–6)

The tests in this series showed that when presented with the two objects described, one at half the distance of the other, the majority of the insects in all cases chose the nearer object. The results indicated that, within the limits imposed by the tests, this choice is not based on the angle subtended by the objects nor is binocular vision necessary. The judging of distance therefore appeared to be made on other criteria than these. A closer study of the orientation behaviour prior to jumping suggested that distance might be estimated during the peering movement.

Table 2 shows the orientations observed in test 1 and is typical of the readings taken in the other tests. The figures for the other tests are therefore not given. The point to note is that before jumping the insects orientated fairly accurately towards the chosen object. From this kind of data a further analysis can be made in terms of the sequence of the orientations and whether or not they are towards the side of the chosen object. The actual angle of orientation is not used. The results of such an analysis for tests 1 and 2 are given in Table 3.

Table 2. *Body orientation while insect peered in a situation with two objects 45 degrees to left and right*

(For further description see text. The orientations were measured with respect to a 0° line midway between both objects. Orientations marked N and D represent orientation to the side of the nearer and more distant object respectively. N and D in the jumping column indicate the object on to which the insect finally jumped.)

Animal	Body orientation to nearest 5°				Jump	
					N	D
1	0°	5° D	90° N	45° N	+	−
	0°	60° N	.	.	+	−
	0°	60° N	45° N	.	+	−
	5° N	10° D	45° N	.	+	−
	45° N	30° D	20° N	45° N	+	−
2	0°	20° N	.	.	+	−
	0°	10° N	30° N	.	+	−
	0°	45° N	.	.	+	−
	5° D	20° N	30° N	.	+	−
	0°	30° N	.	.	+	−
3	0°	45° N	.	.	+	−
	10° D	0°	20° N	.	+	−
	5° N	25° N	.	.	+	−
	0°	10° D	.	.	−	+
	10° N	20° N	.	.	+	−
4	0°	45° N	.	.	+	−
	10° D	90° N	20° N	45° N	+	−
	0°	45° N	.	.	+	−
	0°	45° N	.	.	+	−
	10° N	45° N	.	.	+	−
5	10° D	90° N	45° N	.	+	−
	0°	45° N	.	.	+	−
	5° N	45° N	.	.	+	−
	10° D	40° D	.	.	−	+
	5° N	45° N	.	.	+	−
				Total	23	2

Table 3. *The frequency and order of orientations made prior to jumping on to one of two objects at different distances and at 45° to right and left (tests 1 and 2, Table 1)*

(Orientations: O = along line of 0°; N = to one side of the 0° line, the side of the nearer object, D = to one side of the 0° line, the side of the more distant object. Choice N or D indicates final jump on to near or distant object respectively. 1–5 indicates temporal sequence of orientations.)

Test	Order of orientations															Final choice		
	1			2			3			4			5					
	O	N	D	O	N	D	O	N	D	O	N	D	O	N	D	O	N	D
1	13	7	5	1	19	5	.	9	.	.	3	.	.	.	.	.	23	2
Totals		25			25			9			3			0				
2	14	7	4	.	18	2	.	7	.	.	.	.	.	.	.	.	23	2
Totals		25			20			7			0			0				

There is no significant difference between the numbers of first and second positions (columns 1 and 2), but there is a highly significant difference between the numbers of the second and third positions (columns 2 and 3) (test 1, $P < 0{\cdot}01$, test 2, $0{\cdot}02 > P > 0{\cdot}01$ by χ^2). In other words, the majority of the insects only peer in two positions. Few show a third orientation and fourth orientations are very rare. Approximately half the insects peer first in an unbiased position midway between the two objects. The insects, therefore, 'decide' during the first peering, then turn towards the chosen object, peer in that position and jump.

This behaviour strongly suggested that the insects were measuring the distance to the object by the peering movement.

SECOND EXPERIMENTAL SERIES

Exner (1891), in discussing distance estimation, suggested, among other methods, that for crabs with movable eyes the rate of movement of the image of the object over the eye might be used as a measure of its distance from the animal. The closer the object the faster its image would move over the retina as the eye moved from side to side. Now a locust nymph could achieve exactly the same result by peering. The second series of tests on peering and distance estimation was designed to test this hypothesis directly. The principle was to present the insects with a single object which was moved slightly when they peered at it. By this means it was hoped to confuse the insects as to the distance of the object by increasing or decreasing the relative movement of the image over the retina.

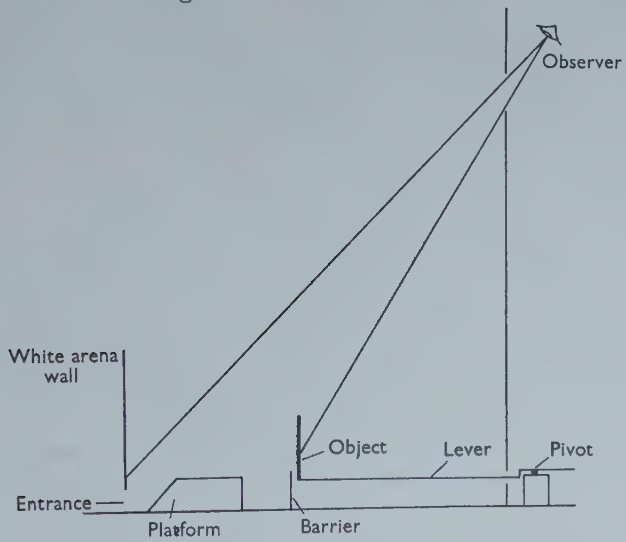

Fig. 4. Elevation of apparatus used in the jumping test with moving object (see text).

Apparatus and method

The apparatus is shown in Fig. 4.

The object was a black rectangle 4 in. tall and 1 in. broad at a distance of 3·5 in. There were two experimental conditions—'moving against' and 'moving with'.

In the former, every time the insect swayed to one side the object was moved slightly in the opposite direction. In the latter the object was moved in the same direction as the animal. Controls were run with the object stationary.

Readings

Note was taken of whether the insect jumped on to the object, overshot it, or jumped short of it.

Results

The results are given in Table 4.

Table 4. *The results of a jumping test with a single moving object*

(For description see text and Fig. 4. The experiment was performed with two groups of animals, the first group being tested under two conditions and the second group being tested under the three conditions shown. S = jumped short, O = overshot.)

Animal	Stationary object				Object moving against insect			
	Hit	Miss	S	O	Hit	Miss	S	O
1	4	1	.	.	0	5	5	.
2	8	1	.	1	1	5	5	.
3	5	0	.	.	0	5	5	.
4	5	0	.	.	0	5	5	.
5	5	0	.	.	0	5	5	.
6	4	2	2	.	0	3	3	.
7	3	2	2	.	0	3	3	.
8	8	0	.	.	0	7	7	.
	42	6	4	1	1	38	38	0

Animal	Object stationary				Object moving with insect				Object moving against insect			
	Hit	Miss	S	O	Hit	Miss	S	O	Hit	Miss	S	O
1	5	0	.	.	5	0	.	.	0	3	3	.
2	5	1	.	1	3	2	.	2	0	3	3	.
3	3	0	.	.	3	2	1	1	0	2	2	.
4	6	1	.	1	3	3	2	1	0	2	2	.
	19	2	.	2	14	7	3	4	0	10	10	.

They showed clearly that when the object was stationary the insects seldom missed it, whereas when it was moved in the opposite direction to the animals' peering motion they misjudged the distance and in almost all cases they jumped short. They never overshot. When the object was moved with the insects the insects managed to jump on to it in most cases. Occasionally they jumped short or overshot.

Second experimental series—discussion

It is to be expected that in the situation 'moving against' the relative motion of the object as perceived by the insect is much greater than it would be for a stationary object at that distance, and is equivalent to the relative motion of a stationary object at a shorter distance from the insect. This is apparently how the insects interpret the information received and would explain why they jump short. This is further

borne out by the fact that the insects were observed to beat the antennae vigorously and to stretch out the first pair of legs and 'paw' the air. This is the behaviour observed in response to a stationary object very close to the insect and probably represents an attempt to climb on to the object.

In the situation 'moving with' the reverse occurs and the object appears to be much further away than it really is. This does not, however, prevent the insects from jumping on to it, since if their direction is correct they will encounter the object during their trajectory. Hence the number of hits in this case is significantly greater than the number of misses. If the direction is not accurate they may overshoot.

By themselves the results of this experiment are not conclusive evidence for a distance-judging method based on relative movement. A binocular method might also be upset by the movement of the object, since the insect might find it difficult to fix the object. Taken in conjunction with the previous tests, however, which showed that binocular vision was not necessary for such a judgement, they appear to support the hypothesis that the image movement produced by peering is the information on which the estimation is made.

It was previously mentioned (p. 512) that when the body moves through a large peering angle the longitudinal axis of the head moves with respect to that of the body. When the experiments reported here were performed there was no available means of measuring this small movement. It was thought possible that the head position was changed so as to minimize the image movement on the retina. Recent high-speed photography has shown that the head movement does not compensate for the body displacement; it results in a lateral movement of the head rather than a radial movement. Thus despite the head movement there is still a large movement of the image on the retina.

GENERAL DISCUSSION

The peering movement which has been described in this paper has been shown to be related to the visual sense. There is evidence to support the idea that it is a method of estimating distance.

Many of the peering angles are small and unaffected by the position of objects in the visual field and it is likely that these movements represent a preliminary scanning. It was observed that they did, in fact, precede the larger biased peering angles. In other words, locusts peer either when looking at something, i.e. measuring the distance of a particular object, or when looking for something, i.e. when scanning the surroundings. On this basis the observation made by Chapman (1955) that locusts peer in a uniform visual field is understandable.

It seems to be generally accepted (Wigglesworth, 1953; Roeder, 1953) that in insects possessing highly developed compound eyes with overlapping frontal fields distance estimation is accomplished by a binocular method. This is based on the work of Baldus (1926) and Friederichs (1931). It is perhaps significant that both these authors worked with predatory insects, *Aeschna* nymphs and cicindelid adults respectively. *Aeschna* nymphs catch their prey by shooting out the labial

mask and cicindelid adults use their large mandibles. In both cases extremely short distances are involved, and while it is clear that in these cases the binocular method is very accurate this accuracy will fall off with distance. In the desert locust there is a fairly large binocular field and thus at short distances the binocular method may be used. At large distances, on the other hand, such as those covered by a locust nymph when jumping, it is possible that the binocular method is no more accurate and perhaps even less accurate than one based on relative movement. It is known that the compound eye may be extremely sensitive to small movements (Burtt & Catton, 1954, 1956), and it is therefore possible that even when objects are at relatively great distances and their image movements correspondingly small the locust may still measure these accurately.

Finally, distance estimation may not be the only function of peering. It is possible that it has a more general function in that the image movements may provide the insect with more information as to the finer details of the visual field.

SUMMARY

1. This paper describes a lateral swaying movement performed by desert locust nymphs. This movement is called 'peering'.

2. The angle through which the body moves is influenced by the position of objects in the visual field, showing that the movement is related to vision.

3. When given a choice of two objects at different distances the nymphs show a preference for the nearer one. The estimation of the relative distances of the two objects is not achieved by a binocular method nor is it based on the angle subtended by the objects.

4. An experiment is described in which an object is moved while the insect is peering. If the object is moved in the opposite direction to the insect's motion the insect jumps short of the object. This seems to support the hypothesis that one of the functions of peering is to estimate distance by the extent of the movement over the retina of an object's image.

5. This method of distance estimation is discussed with relation to the binocular method.

6. It is suggested that in some cases the peering observed may represent a preliminary scanning of the visual field and may provide information about the finer details of the field.

The experiments reported in this paper were performed during the tenure of grants from the Carnegie and Cross Trusts and formed part of a thesis for the degree of Ph.D. of St Andrews University. The work was carried out in the Department of Entomology, Oxford, and I wish to thank Prof. G. C. Varley for his kindness in affording me facilities. I am grateful to Dr B. P. Uvarov, F.R.S., and Dr T. H. C. Taylor of the Anti-Locust Research Centre for their encouragement at all stages. I also wish to thank Dr F. L. Waterhouse, Queen's College, Dundee, for criticism and encouragement. I am grateful to Dr D. M. Vowles, Psychology Department, Reading, and to my wife for their criticism of the manuscript.

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LONG-TERM FACILITATION IN A SWIMMING SEA ANEMONE

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(*Received* 13 *March* 1959)

The peculiar behaviour of the anemone, *Stomphia coccinea* (O. F. Müller), in response to certain starfishes was noted by Yentsch & Pierce (1955). More detailed description of the swimming reaction and attempts to identify the stimulating factor involved were made by Sund (1958). The easily measured sequence of events which constitutes the response provides an opportunity to analyse quantitatively integrated behaviour in a coelenterate. This study is an attempt to identify and measure a process which might be considered a type of learning in an animal with simple neural organization. Guessing that the relationship between the starfish *Dermasterias* and *Stomphia* might be a predator–prey one (although no evidence favours this assumption) it seemed likely that encounters which resulted in no harm to the anemone might result in habituation. Experiments have therefore been done to test whether the swimming behaviour of this animal is modified by previous occurrences of the stimulus. It was a surprise to find that the reaction is enhanced by repetition. The results indicate long-lasting changes within the animal which have been called here *long-term facilitation*. This is a kind of modification of behaviour of the same apparent complexity as habituation but with opposite sign. Facilitation of monosynaptic reflexes lasting minutes or hours has been mentioned by Eccles (1958), but while such relatively simple neural mechanism for modifiability of response has been demonstrated, no comparable type of learning is currently included in several reviews of animal behaviour, though it is recognized that a facilitation process could be the basis of imprinting. In the attacking response of young cephalopods Wells (1958) has demonstrated facilitation without reward.

The experiments were performed during the summer of 1958 at the Friday Harbor Marine Laboratory of the University of Washington.

DESCRIPTION OF THE SWIMMING ACTIVITY

Sund (1958) gives an illustrated account of the swimming reaction. A brief description is presented here, with a few additional observations based on the differences between the small specimens used in this study and the large ones used by Sund and upon measurements of the duration of the various components of the behaviour.

Following appropriate stimulation *Stomphia coccinea* successively retracts, closes, elongates, opens widely, detaches itself from the substrate, and bends from side to side violently, propelling itself through the water. During the swimming, and for

some time after, the animal usually fails to respond to external stimulation, either mechanical or chemical. The swimming reaction may be initiated by contact with any of several species of starfishes or by appropriate electrical stimulation. The intensity and duration of the response do not depend on stimulus strength, above threshold.

The initial withdrawal is probably not truly a part of the reaction, but only a tactile response. It does not occur in specimens which are induced to swim shortly following one swimming period. Gentle touch by a specimen of the starfish, *Dermasterias imbricata* (Grube), can result in swimming without initial closure.

The bending movements which effect locomotion usually follow one another at a regular frequency of one or two per second, but the direction of successive movements is often irregular, there being no apparent relationship between one movement and another in space but only in time. In large specimens the bending may at first take the form of a smooth progression of contraction around the circumference of the animal, the result being a swirling of the oral end. Swirling movements were never seen in small specimens. In large specimens the bending (or swirling) movements begin before detachment and probably aid in loosening the foot. This is generally not true for small individuals in which detachment is accomplished by contraction of the basal region only. The frequency of the bending is lower in small specimens so that their reaction is less vigorous and does not result in propulsion.

The period of swimming, here taken as the interval between the first sign of the whole response (extension of the column) and the last swimming movement, is quite variable but typically lasts about 5 min. The duration decreases only after several trials. More immediate effects of repetition are longer latency of the first movement and decrease in the frequency of the swimming movements.

The unresponsive period is even more variable. It may begin during the swimming or some time afterward. Its duration varies from immeasurably short to several hours. On successive trials this duration may either increase or decrease. In one individual, stimulated to fatigue, the period of unresponsiveness grew for the first several trials and then decreased to extinction, and on the next 2 days swimming was not accompanied by any refractoriness. The refractoriness is apparently never complete. A very strong stimulus such as heavy prodding with a rod or cutting the animal in two interrupts the swimming. The cessation is abrupt and may be accompanied by a partial or total withdrawal response. After about half a minute the swimming resumes.

Partial reactions are occasionally observed. Rarely, subthreshold stimulation (see later) produces abortive responses in which the reaction ends after detachment. In the tiniest specimens and in some fatigued ones the whole sequence may occur minus the swimming contractions themselves. Some individuals may swim without opening the oral disk. Others are seen to produce lateral contractions without elongation of the column and detachment.

Parts of animals show the swimming behaviour. The basal halves of animals divided across the oral–aboral axis show all the features of the response normal for them, including long-term facilitation (see later) and resumption of activity after

interruption. The oral pieces do not survive well, but the few observations on them suggest that they also retain the ability to make specific response to the starfish.

The sequences in which elements such as elongation, opening, bending, detachment, or refractoriness are missing, or rearranged, as in the ordering of detachment and bending, together with the possibility of resumption of the sequence after interruption, suggest that the swimming of *Stomphia* is not organized as a simple chain of reflexes such as might explain the walking of *Hydra*. The normal response seems to be an integrated activity of the whole animal in which functional subdivisions are normally linked by a central cause, but are not sequentially dependent. Furthermore, it appears that during a period of interruption in which some other activity takes place some part of the animal is able to store the information which constitutes the internal stimulus for swimming.

EFFECT OF REPETITION OF STIMULUS

When stimulated by the starfish, *Dermasterias*, the response is labile and facilitated by previous occurrence of the stimulus. Many individuals, especially small ones, do not react to starfish on the first contact except by an ordinary tactile withdrawal. Stimulation every few minutes (or as soon as the animal opens) usually results in a swimming response on the first, second, or third repetition, but some specimens require more trials and a few do not react with over 20 repetitions. The facilitation is not erased by a response. It decays very slowly and in some cases outlasts the maximum duration of test, which was 7 days. Since the smallest specimens (6–8 mm. in diameter) do not swim and large ones require little facilitation, one can suppose the ontogeny of the behaviour to involve a gradual decrease in the threshold for response. Lacking a knowledge of the experience of individuals it is not possible to decide whether this occurs without encountering the stimulating starfish.

A standard stimulation by means of electric shock allows more precise characterization of the facilitation. The following list summarizes the tests made in order to decide upon a standard stimulus. Pulse stimuli produced by a Grass S4 stimulator were delivered through stimulating electrodes placed against the column. Tests were carried out at about 20° C. Eighteen animals were used in all. The small number of specimens available and the change of reactivity with experience prevented extensive testing. The useful information was gathered with as few trials as judgement allowed:

(*a*) There is ordinarily no response to the first shock.

(*b*) A retraction and closure occurs following the second shock. This facilitation is presumed to be the same neuromuscular facilitation common to other actinians; it lasts 3 sec.

(*c*) In open and not previously stimulated animals a train of precisely 8 shocks results in a full swimming response. The frequency may be any value between 1/2 sec and 4/sec; the threshold is the same as for (*b*). The figure 8 was observed in all of the sixteen specimens which did swim; fewer shocks never released swimming in one to three widely separated trials on each of 8 fresh or long-rested animals; more were

rarely required. In trials in which 8 shocks did not produce swimming additional shocks usually failed as well.

(*d*) With a stimulation frequency of 1/sec. or greater the latency of the response measured from the first shock is nearly constant at 8 sec.

(*e*) At frequencies less than 1/3 sec., the response does not occur even after many repetitions.

(*f*) At frequencies greater than 4/sec., more than 8 shocks are required.

The following tests employed a stimulus of 8 shocks at 1/sec., each shock of 0·1 msec. duration and just above the voltage threshold for a neuromuscular response on the second shock. It should be stressed that the number of shocks necessary is quite constant in animals which have not been tested previously. This is true even of those which do not react readily to *Dermasterias*. Some closed animals, however, do not react even to many shocks.

In the main facilitation experiments a burst of 7 shocks was delivered. This usually results only in the closure and retraction responses. But the same stimulus repeated 1–5 min. later causes a full swimming response. With repetition of this sequence the interval between the conditioning and test stimuli can be increased to hours or even days so that a specimen always responds to 7 shocks. An individual which has been thus conditioned may be made to respond to only 6 shocks by similar conditioning. Such an individual may retain the ability to react to 6 shocks after at least 1 week without practice. This decrease in threshold for the swimming response, lasting hours or days, was found in each of 8 specimens. Facilitation lasting 1 week was tested in two specimens only. The maximum period was not determined. The lowered number of stimuli required was probably not due to change of other conditions since the previous stimulation, because 12 animals kept under the same conditions for the same length of time or longer before their first electrical stimulation required a full 8 shocks to release swimming.

EFFECTS OF MIXED STIMULATION

For anemones which do not react the first time contact with *Dermasterias* may be considered a subthreshold stimulus. If this follows, or is followed within a few minutes by, a subthreshold electric stimulus (5 to 7 shocks) the two kinds of stimulus may sum and produce a response. This is true even of anemones which cannot be made to swim after a score of trials with starfish alone. Subsequent to a single response elicited by mixed stimuli such individuals acquire an ability to respond to a starfish alone. These experiments can be arranged to look like Pavlovian conditioning. The difference is that the 'conditioned' stimulus is not a neutral one, but a stimulus which ordinarily produces the response in other members of the species without prior conditioning. The simplest explanation of this acquired behaviour is not that there has been a closure of neural paths involved in two different reactions but that there has been a lasting decrease in the threshold for a response that is probably inherent.

DISCUSSION

A difficulty in describing this behaviour in *Stomphia* as learning arises in the attempt to assess adaptiveness of the response. *Dermasterias* is not known to feed upon anemones even when there is opportunity to do so. Even a 'hungry' starfish, which subsequently devoured chitons, crept over anemones which were fixed in place without apparent interest in them as food. The distribution and habitat of *Stomphia* and *Dermasterias* are such that they would rarely, if ever, contact each other in nature. Sund (1958) discussed other possible significance for the response than predator escape, but he found no evidence to support alternative hypotheses. It remains possible that the adaptiveness of the response must be sought in the history of the species and that at present it is a behavioural atavism. Even this suggestion, however, does not provide escape from the conclusion that the 'learning' has no adaptive significance for the individual. An alternative answer to the question of adaptiveness is that the response is a non-specific one to a predatory group. The negative results of tests made by the previous workers using other animals including many echinoderms from the Puget Sound area weaken the apparent reasonableness of this suggestion. It seems that the response is quite specific to at most a small group of echinoderms. Perhaps the clue to this relationship must be sought in some other portion of the range of the species.

Other cases of 'learning' in actinians, both habituation and associative learning, requiring memory lasting for periods similar in length to those found here have been reported (reviewed in Thorpe, 1956). Short-term 'memory' perhaps not involving persistence for days is suggested by the results of Batham & Pantin (1950), who found that anemones might walk away hours after a period of intense stimulation. The present results do not add to a knowledge of the mechanism of memory in coelenterates, but they do point to the possibility of such an analysis.

The two kinds of stimulus used in this study probably affect directly different structures in the organism. The identity of intensity threshold for electrical stimulation in both simple withdrawal response and the swimming response suggest that the same structures are initially stimulated. In other species of anemones the withdrawal response is due to stimulation of a through-conducting nerve net. A peculiarity of elicitation of swimming via this through-conducting net is the rather large and constant number of pulses necessary. A similar phenomenon occurs in *Calliactis* in which slow contractions are produced after several shocks ('usually not less than six') by low-frequency stimulation of the through-conducting net (Ross, 1957). These slow contractions are of nearly constant latency regardless of frequency, but are graded in intensity by frequency and number. The present case differs notably in that the frequency must be great enough to produce the fast muscle response. It is possible that the swimming is initiated by slow-type activation of certain muscles.

The great specificity of the response to other organisms shows that stimulation by starfish is routed through sensory levels and that special sensory mechanisms must be present. Stimulation by the starfish presumably does not involve the through-

conducting nerve net, for in some cases the swimming response can be elicited without a withdrawal response. The long-term facilitation which occurs must take place along the functional path between this nerve net and the reacting muscles. Since both shock and starfish stimulation produce the response and because subthreshold stimulation by each can summate, the pathways followed by excitation from each must clearly overlap. This overlap may or may not involve more than the last stage, the muscles themselves. The facilitation must occur at the initial point of overlap since electrically induced reactions result in marked decrease in threshold to starfish only when the two are presented in the same trial. These considerations place the probable location of the long-term facilitation at the point of convergence of the two kinds of stimulation used and somewhere between the through-conducting nerve net and the responding muscles. The neuromuscular junction may be named as a possible site.

The decreased latency of response that Wells found in *Sepia* and the change of behaviour of *Stomphia* seem likely to be based on similar mechanisms. The first might involve an increased efficiency in the handling of information necessary for recognition of mysids, but the spread in the range of releasing stimuli suggests a decrease in the threshold for attack. The two cases operate in quite different parts of the receptor-effector chain. Wells locates the process in *Sepia* 'somewhere between the retina and the motor centres', whereas it takes place at or near the muscles in *Stomphia*.

SUMMARY

1. Repetition-produced modifications in the behaviour of the swimming sea anemone, *Stomphia coccinea*, are described. Lowered threshold to number of electrical shocks on successive trials indicates a kind of 'learning' called here *long-term facilitation*.

2. Dissection of the behaviour into its components, both by experimental techniques and observation of atypical cases, shows the swimming reaction not to be simply a chain of reflexes, but to be 'centrally' co-ordinated.

3. The conditions for electrical elicitation of swimming are shocks sufficient in intensity to activate the through-conducting nerve net repeated eight times in the frequency range of 1/2 sec. to 4/sec. Fewer than 8 shocks constitute a subthreshold stimulus in fresh animals; more than 8 are rarely required.

4. Repeated subthreshold stimulation by starfish or by 7 electric shocks result in a long-lasting facilitated state in which the same stimulus repeated hours later may produce a full response. The facilitated condition has been observed to last 7 days. Controls kept without stimulation do not show facilitation.

5. The probable site of this long-term facilitation is discussed. It is suggested that this site is at the point of convergence of the two types of stimulation used and between the through-conducting nerve net and the responding muscles.

The author wishes to thank Dr D. L. Ray and Dr A. H. Whiteley for making possible this study, and Dr T. H. Bullock for reading of the manuscript. The work

was performed during the tenure of a National Science Foundation predoctoral Fellowship and was aided by a grant (B21) to Dr T. H. Bullock from the National Institute of Neurological Diseases and Blindness.

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AMINO ACID ABSORPTION IN THE LOCUST (*SCHISTOCERCA GREGARIA* FORSK.)

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(*Received* 23 *March* 1959)

INTRODUCTION

Insects are known to possess an exceedingly high concentration of amino acids in the haemolymph, probably higher than in any other animal group (cf. Buck, 1953). On the other hand, recent work has shown that in the locust the mid-gut region is permeable to the relatively large monosaccharide molecules which appear to be absorbed largely by facilitated diffusion (Treherne, 1958*a*, *c*). With such an apparently permeable absorptive surface the back diffusion of the rather small rapidly diffusing amino-acid molecules might be expected to present certain difficulties in their absorption. Furthermore, most membranes possessing secretory or transport mechanisms for non-electrolytes appear to be relatively impermeable to passively diffusing molecules (Wilbrandt, 1954) and it is, therefore, of interest to know something of the mechanism of amino-acid absorption in the locust. In the present investigation an attempt has been made to throw some light on these processes by following the uptake from, and the concentration changes occurring within, the lumen of the alimentary canal of *Schistocerca gregaria* (Forsk.).

METHODS

All experiments were carried out on adult female *Schistocerca gregaria* (Forsk.) which had been deprived of food for the preceding 24 hr. They were reared and maintained at $28 \pm 1{\cdot}0^\circ$ C. and fed on bran and on fresh green wheat grown in pots.

The absorption of amino acids was studied using some techniques previously employed in investigations on sugar absorption in insects (Treherne, 1957, 1958*a*). The various experimental solutions used were injected into the alimentary canal by means of a fine nylon hypodermic needle which was thrust into the rectum of a CO_2-anaesthetized insect and sealed into position with wax. In each case 0·15 ml. was used, which was sufficient to fill the whole of the mid-gut and hind-gut. To determine the major sites of absorption in the alimentary canal the gut lumen was filled with a solution containing ^{14}C-labelled amino acid together with the dye Amaranth (Azo-Rubin S) which has been shown not to be absorbed from the gut lumen in this insect. After an appropriate period the gut was excised, quickly washed in saline, ligatured and then cut into appropriate portions which were dropped into a suitable volume of a solution buffered at pH 10·0. The net percentage absorption was calculated from the ratio of radioactive amino acid to dye in the various parts of the

alimentary canal. The concentration of Amaranth used in these experiments was 0·04 M/1. Dye concentration was determined in solution at pH 10·0 using a Unicam absorptiometer at an absorption maximum of 510μu. The radioactivity of the ^{14}C-labelled compounds was assayed in solution using a thin-windowed G.M. tube (G.E.C. CV 2139) as previously described (Treherne, 1957) or on a planchette after diluting the radioactive fluid to a standard volume (0·1 ml.) and evaporating to dryness.

In some further experiments the concentrations of glycine and serine, the radioactivity and the fluid volume in the mid-gut caeca were followed over a period of 45 min. To collect samples of the experimental solution from the lumen of the caeca the whole gut was removed from a CO_2-anaesthetized insect and the caeca were isolated by ligatures. The whole gut was then very quickly washed in distilled water and dried with filter-paper. The mid-gut caeca were punctured over a waxed slide and the fluid was collected for analysis in a 5·0μl. waxed micropipette. The volume changes were followed using ^{131}I labelled albumen as a volume indicator (Phillips, unpublished). The amount of albumen added to the solution was so small that it did not have a detectable effect on the osmotic pressure. Tests showed that the radioactivity of ^{131}I in the haemolymph was only 1·6% of that in the gut lumen, after a period of 45 min., showing that only a very small amount of the labelled material was lost from the lumen during this period.

The free amino acids in the haemolymph and in the gut fluids were separated and identified by paper chromatography. The freshly collected haemolymph was precipitated with two volumes of absolute ethanol, dried *in vacuo* over P_2O_5 and then restored to twice its original volume by the addition of 60% ethanol (Raper & Shaw, 1948). Successive 5–10μl. volumes of this fluid (or mid-gut fluid) were then transferred to Whatman no. 1 filter-paper for separation by two-dimensional descending chromatography. The solvent systems used were 70% *n*-propanol followed by water-saturated phenol (Chen, 1958) and *n*-butanol/acetic acid/water (74/19/50) followed by phenol (Fowden & Steward, 1957). The detection spray used contained 0·4% ninhydrin and 0·2% cobalt chloride in isopropanol (Wiggins & Williams, 1952). For quantitative determination of amino acids the papers were treated with the modified ninhydrin spray of Connell, Dixon & Hanes (1955). The amino-acid spots were cut out, sprayed with 0·05 M borate buffer at pH 9·0 in methanol to remove ammonia and then estimated by the procedure of Cocking & Yemm (1954).

Osmotic pressure determinations on haemolymph and experimental solutions were made by the technique of Ramsay (1949) as modified by Ramsay & Brown (1955).

RESULTS

The chromatographic analysis of the haemolymph revealed the presence of ten amino acids: glycine, alanine, valine, leucine (and/or isoleucine), proline, tyrosine, serine, threonine, glutamic acid and histidine. In addition appreciable amounts of glutamine were also detected. Fig. 1 is a diagram of the distribution of these compounds on a chromatogram developed with 70% *n*-propanol followed by phenol.

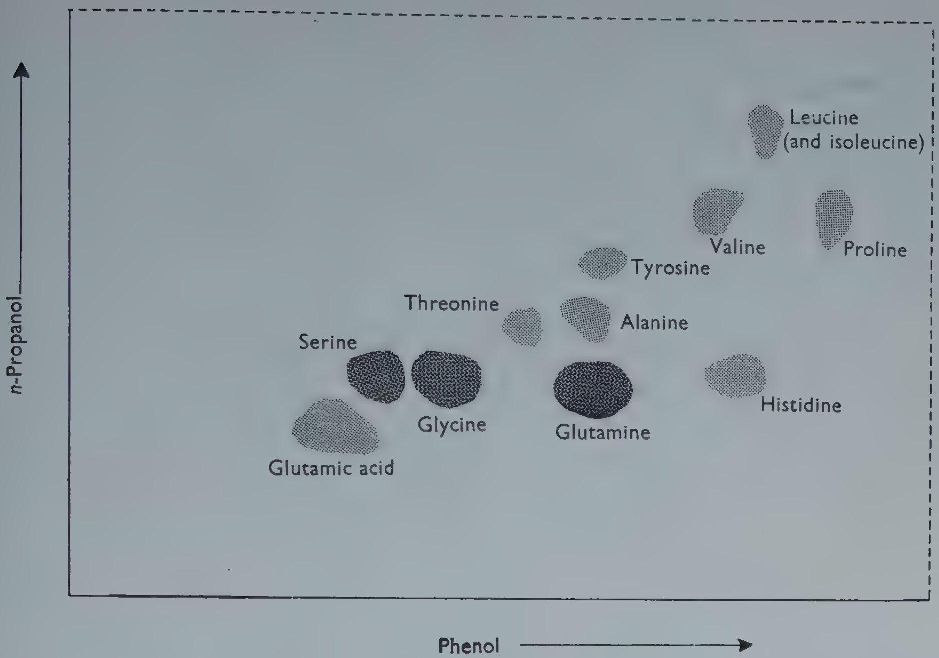

Fig. 1. The distribution of amino acids and glutamine on two-dimensional chromatograms developed with 70 % *n*-propanol followed by phenol.

Table 1. *The concentrations, in* mM./l., *of amino acids and glutamine in the haemolymph*

Amino acid	Experiment no.										Mean ± S.D.
	1	2	3	4	5	6	7	8	9	10	
Glycine	20·0	31·5	39·0	39·5	37·4	28·8	30·5	42·5	36·0	36·8	33·2 ± 7·1
Alanine	0·8	8·0	5·7	3·9	4·6	4·4	1·1	2·8	4·6	1·5	3·7 ± 2·2
Valine	0·8	2·2	4·7	6·2	6·3	4·4	10·9	6·0	6·5	1·9	5·0 ± 2·9
Leucine (and/or isoleucine)	12·7	0·8	1·6	1·1	5·2	0·5	0·7	2·2	0·8	0·4	2·6 ± 3·8
Proline	7·3	1·9	4·2	2·1	4·9	1·4	2·2	7·3	7·2	1·4	4·0 ± 2·5
Tyrosine	1·1	1·7	0·7	3·3	5·1	3·3	3·4	4·3	1·5	1·0	2·5 ± 1 5
Serine	28·7	30·0	50·0	31·5	30·0	33·3	30·5	41·5	27·5	43·5	34·6 ± 2·3
Threonine	1·7	0·9	2·1	3·7	3·0	2·1	2·1	4·7	1·0	1·5	2·3 ± 1·3
Glutamic acid	5·9	6·2	5·6	4·7	8·0	0·5	3·0	6·5	8·8	2·3	5·1 ± 2·6
Histidine	0	0	2·2	3·0	0·6	0·5	0	1·5	2·7	0	1·0 ± 1·1
Glutamine	10·3	8·9	13·5	10·5	13·1	12·6	11·2	9·8	8·8	10·7	10·9 ± 1·6

The concentration of these amino acids and glutamine are tabulated in Table 1. These results showed that glycine and serine were present in relatively high concentration, 33·2 and 34·6 mM/l. respectively. The remaining eight amino acids were found to be at very much lower concentrations, varying between 1·05 mM/l. for histidine and 5·1 mM/l. for glutamic acid. The concentration of glutamine averaged 10·9 mM./l. Despite the fact that the insects used in these experiments were taken

from the same batch there was a great deal of individual variation in these results. This is particularly evident with the results for leucine (and isoleucine) in which values varied between 0·5 and 12·7 mM./l. The results for alanine and valine were also characterized by a high degree of variability. The concentrations of glycine and serine, and also of glutamine, exhibited much less individual variation, to an extent which cannot be attributed to a greater degree of accuracy in their determination at higher concentration.

The freezing-point depression of the haemolymph of adult females was also measured and is summarized in Table 2.

Table 2. *The freezing-point depression of the haemolymph*

Serial	Δ °C.	Mean
1	0·770	
2	0·755	
3	0·795	
4	0·760	0·762
5	0·745	
6	0·750	
7	0·760	

For injection into the gut lumen an experimental solution was devised in which the concentrations of amino acids and other substances were as close as possible to those of the haemolymph. The substances incorporated in this solution are tabulated in column I of Table 3. The amino acids were present in concentrations as measured in the present investigation, while the sugar concentrations have been taken from the data of Treherne (1958*c*). The salt concentrations were devised by maintaining the concentration of cations given by Duchâteau, Florkin & Leclercq (1953), the anions being in the same proportion as in Hoyle's balanced salt solution (Hoyle, 1953). This gave a total osmotic pressure of $\Delta = 0{\cdot}790°$ C., which was slightly in excess of that of the haemolymph. The dye Amaranth was incorporated in the solution, without unduly raising the osmotic pressure, by a reduction of the total salt concentration as shown in column II of Table 3. This solution had an osmotic pressure of $\Delta = 0{\cdot}760°$ C. which approximated to that of the haemolymph.

The concentrations of glycine and serine in the haemolymph showed least variation and because of their high and relatively constant concentrations these amino acids were selected for the study of absorption from the gut lumen. The first experiments were concerned with the absorption of ^{14}C-labelled glycine and serine in order to determine the sites of absorption in the alimentary canal. The concentrations of glycine and serine in the injected solution were similar to their concentrations in the haemolymph (i.e. 33·2 and 34·6 mM./l. respectively), the remaining substances being as in column II of Table 3. The results of these experiments are summarized in Fig. 2 from which it will be seen that both glycine and serine were absorbed most rapidly from the mid-gut caeca. After 15 min. 18·1 % of the labelled glycine and 14·8 % of the labelled serine had been absorbed from the caeca, while in the remaining parts of the gut the absorption occurred much less rapidly. After

1·0 hr. 57·7% of the labelled glycine and 63·1% of the labelled serine had been absorbed from the caeca, with a less rapid absorption from the ventriculus and a slight absorption from the hind-gut.

Table 3. *The composition of the two experimental solutions used in the investigation*

Substance	Solution I	Solution II
Glycine	33·2* mM./l.	
Alanine	3·7	
Valine	5·0	
Leucine	2·6	
Proline	4·0	
Tyrosine	2·5	
Serine	34·6*	
Threonine	2·3	
Glutamic acid	5·1	
Histidine	1·0	
Glutamine	10·9	
Glucose	0·94	
Trehalose	20·30	
NaCl	73·7	—
NaH_2PO_4	7·6	19·4
$KHCO_3$	5·05	13·5
$CaCl_2$	17·80	—
$MgCl_2$	34·60	—
Amaranth	—	40·0
Δ °C.	0·790	0·760
pH	6·5	7·0

* Generally labelled with ^{14}C in some experiments.

In Fig. 3 the absorption of labelled glycine is compared with that of labelled serine by plotting the percentage of the radioactive amino acids remaining in the caeca on a logarithmic scale against time. It will be seen that for both substances the rate of absorption from the caeca is approximately the same and falls off exponentially with time.

The changes in concentrations of glycine and serine in the gut following injection of the experimental solution (column I, Table 3) into the gut lumen were studied by separating the contents of the caeca on two-dimensional chromatograms and determining the concentrations of these two amino acids. The concentration of glutamine in the lumen of the caeca was also followed in this manner. It seemed possible that fluid already present in the gut lumen might significantly affect the concentration of the substances in the experimental solution. To test this possibility the fluid in the caeca was removed immediately after the experimental solution had been injected via the rectum and the concentrations of glycine, serine and glutamine determined. The results are summarized in Table 4, from which it will be seen that their concentrations in the fluid recovered from the caeca did not differ significantly from their concentrations in the experimental solution.

Experiments were also carried out in which the concentrations and radioactivity of ^{14}C-labelled glycine and serine were followed after injection into the gut lumen. These results are illustrated in Figs. 4 and 5. With glycine (Fig. 4) the concentration

in the lumen tended to rise above that of the haemolymph. After 15 min. the concentration in the lumen averaged 40·5 mM./l. as against 33·2 mM./l., in the haemolymph, a difference which was not significant ($P < 0·1$). After 30 min. the concentration averaged 45·5 mM./l. and after 45 min. was 43·4 mM./l., both being significantly different from that of the haemolymph ($P < 0·01$ and $< 0·02$ respectively). During this time the concentration of the labelled glycine in the caeca lumen fell relatively rapidly reaching a mean value of 16·5 mM./l. after 45 min.

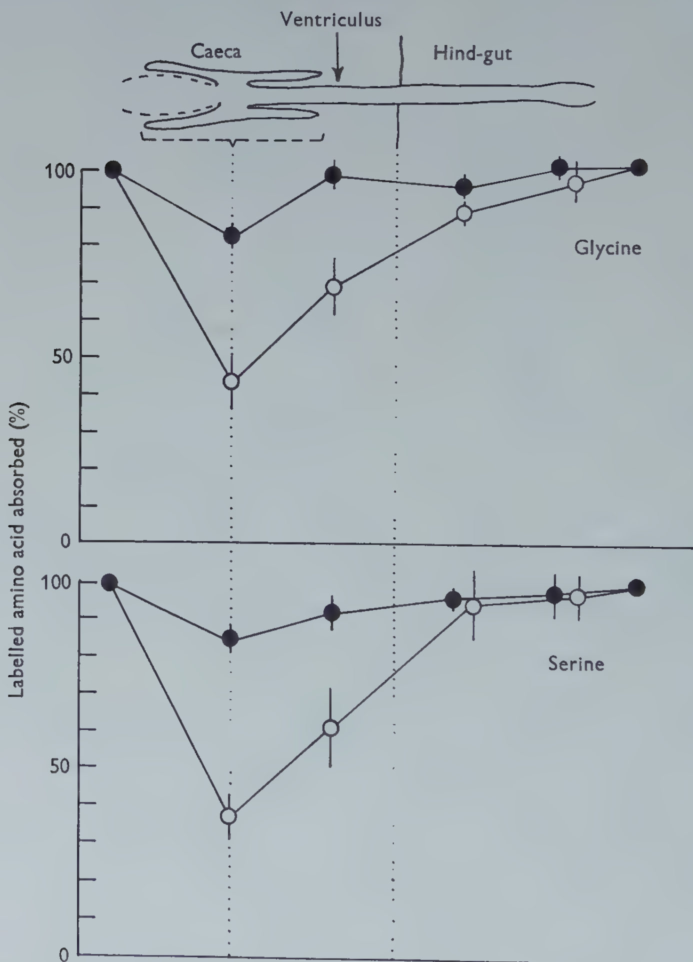

Fig. 2. The percentage absorption of ^{14}C-labelled glycine and serine from the alimentary canal after 15 min. (closed circles) and 60 min. (open circles). The vertical lines illustrate the extent of the standard deviation.

The concentration of serine (Fig. 5) in the caecal fluid also showed a slow increase after injection of the experimental solution into the gut lumen. The concentrations after 15·0 and 30·0 min. were not statistically different from that of the haemolymph

($P<0{\cdot}3$ and $<0{\cdot}10$ respectively). After 45 min., however, the concentration in the lumen averaged 45·3 mM./l. which was significantly different from that of the haemolymph ($P<0{\cdot}02$). During this time there was a progressive rapid fall in the concentration of the ^{14}C-labelled serine in the lumen.

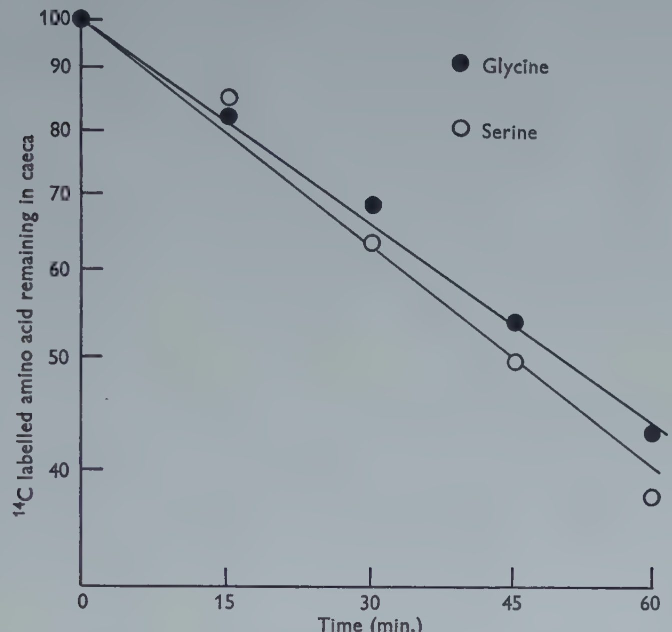

Fig. 3. The percentage of ^{14}C-labelled glycine and serine remaining in the mid-gut caeca plotted on a logarithmic scale against time.

Table 4. *The concentrations of glycine, serine and glutamine in the fluid recovered from the caeca immediately after injection of the experimental solution into the gut lumen*

Serial	Concentration (mM./l.)		
	Glycine	Serine	Glutamine
1	32·7	37·5	10·3
2	35·0	31·5	12·7
3	32·7	38·5	12·0
4	35·0	37·3	10·5
5	34·0	36·0	13·0
Mean ± S.D.	33·9 ± 1·15	36·1 ± 2·54	11·7 ± 1·24
Initial concentration	33·2	34·6	10·9

With glutamine the mean concentration in the lumen of the caeca also showed a steady rise, to a value of 15·3 mM./l. which was significantly higher than the value of 10·9 mM./l. for the haemolymph ($P<0{\cdot}001$).

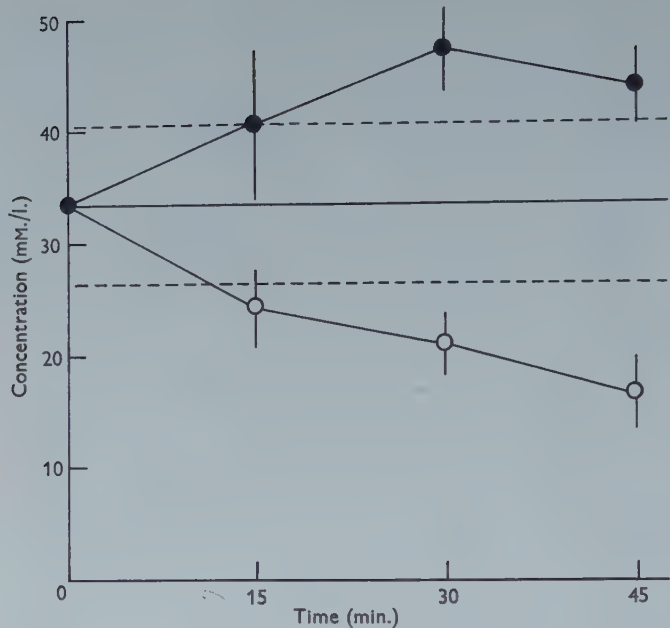

Fig. 4. The changes in concentration of total glycine (closed circles) and ¹⁴C-labelled glycine (open circles) in the caeca following the injection of experimental fluid into the gut lumen. The vertical lines through the points represent twice the standard error of the mean. The three horizontal lines represent the mean and twice the standard error of the initial glycine concentration in the haemolymph.

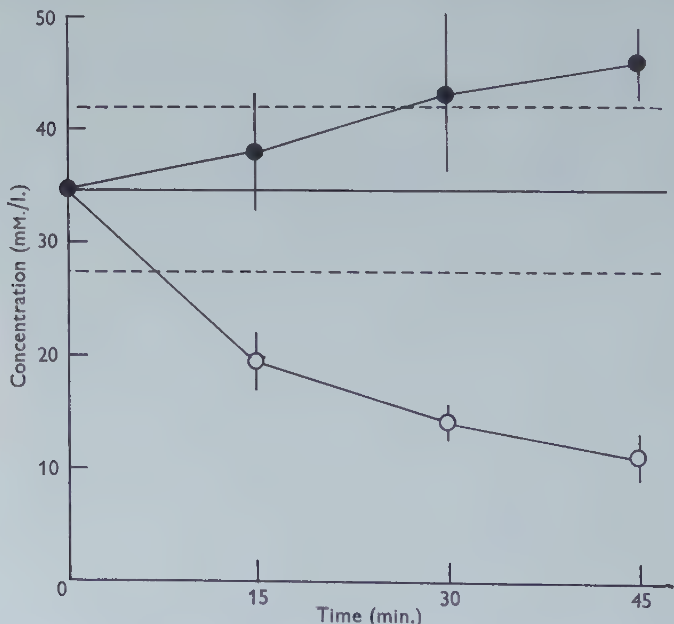

Fig. 5. The changes in concentration of total serine (closed circles) and ¹⁴C-labelled serine (open circles) in the caeca. Other symbols as in Fig. 4.

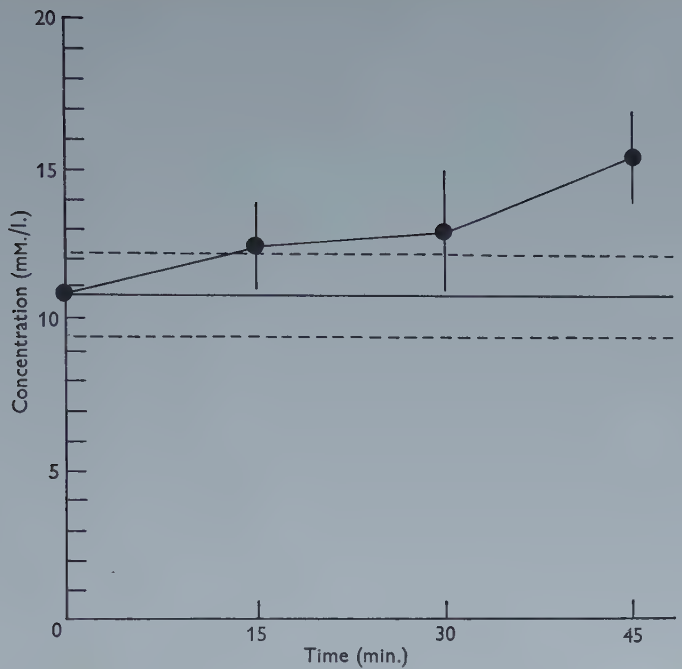

Fig. 6. The concentration of glutamine in the caeca following the injection of the experimental solution into the gut lumen. Other symbols as in Fig. 4.

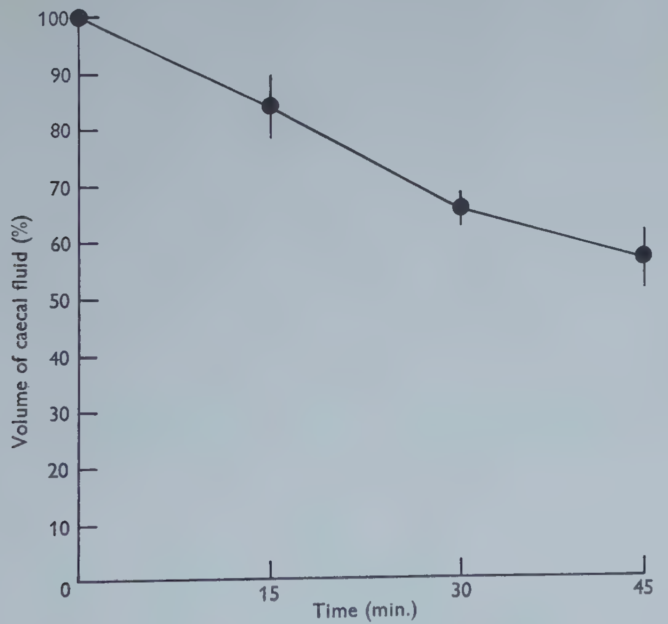

Fig. 7. The changes in volume of the caecal fluid, followed using ^{131}I-labelled albumen as a volume indicator. The vertical lines represent the extent of the standard deviation.

The change in volume of the caecal contents is illustrated in Fig. 7. The volume showed a progressive fall to 57% of the original volume after 45 min.

Using the data illustrated in Fig. 7 it is possible to calculate the rate of disappearance of glycine and serine from the lumen of the caeca. In Fig. 8 the percentage of each amino acid remaining in the lumen has been plotted on a logarithmic scale against time. These results illustrate the very slow net absorption of the amino acids as compared with the fast absorption of the ^{14}C-labelled molecules.

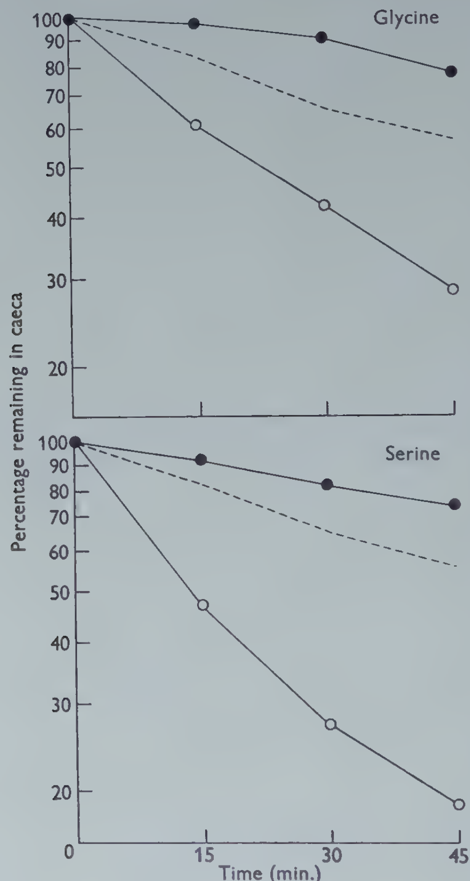

Fig. 8. The percentage of total amino acids (closed circles) and ^{14}C-labelled amino acids (open circles) remaining in the caeca plotted on a logarithmic scale against time. The broken lines represent the percentage of water in the caeca.

DISCUSSION

The amino acids in the haemolymph of *Schistocerca gregaria* are in general similar to those found in many other insects. All of the amino acids in the present investigation were found to occur in the haemolymph of a wide variety of insects by Duchâteau & Florkin (1958), with the exception of serine which these authors do not mention. There is also qualitative agreement with the amino-acid picture in the

haemolymph of *Aeschna* (Raper & Shaw, 1948), *Bombyx mori* (Wyatt, Lougheed & Wyatt, 1956) and *Culex pipiens* (Chen, 1958).

Quantitatively the haemolymph of *Schistocerca* is characterized by high concentrations of glycine and serine, and also of glutamine, and is in this respect similar to that of the larva of *Bombyx mori* (Wyatt *et al.* 1956). The high concentration of these substances in the haemolymph is not apparently characteristic for all insects however—in *Hydrophilus*, *Gasterophilus* and *Apis*, for example, they occur in relatively low concentrations (Duchâteau & Florkin, 1958). The strikingly large degree of individual variation obtained in the concentrations of certain amino acids in *Schistocerca*, especially leucine, valine and alanine, has also been recorded in other insects. Wyatt *et al.* (1956), for example, quote values for the concentrations of proline, tyrosine and histidine in which the variation exceeded an order of magnitude.

The absorption of ^{14}C-labelled glycine and serine took place largely in the mid-gut region and occurred most rapidly from the caeca. The disappearance of the amino acids from the ventriculus may not have been due entirely to an absorption by this organ, however, for it is possible that some may have diffused into the lumen of the caeca as the concentration there fell. The uptake of other nutrient substances has also been found to occur rapidly from the mid-gut caeca. In *Periplaneta americana* the uptake of glucose (Treherne, 1957) and triglyceride (Treherne, 1958*d*) occurred most rapidly in this region; in *Schistocerca* monosaccharides were also rapidly absorbed from the caeca (Treherne, 1958*b*, *c*).

The composition of the experimental solution was adjusted so that the concentrations of the amino acids and other substances, and also its osmotic pressure, were similar to those in the haemolymph. The results showed that there was a definite rise in the concentrations of glycine and serine in the caeca, above their concentrations in the haemolymph, following injection of the experimental solution into the caeca. With glutamine also there was a slow increase in concentration. This effect resulted from the demonstrated removal of water, a process which occurred more rapidly than the net transfer of the amino acids across the gut wall (Fig. 8). Absorption of these amino acids could, therefore, be brought about as a result of a diffusion gradient thus established, the transfer of glycine and serine being dependent, in part at least, upon the net movement of water molecules into the haemolymph. These water movements could be produced either by an active transport of water or by a passive movement resulting from net ion movements into the haemolymph (cf. Robinson, 1954). In the absence of any demonstrated accumulation of amino acids against concentration gradients it seems likely that active uptake of glycine and serine would form only a relatively small part of the absorptive mechanism in the caeca of this insect. The absorption of these amino acids by the locust would thus seem to be fundamentally different from the processes of absorption in mammals. Wiseman (1955, 1956), for example, has demonstrated for several amino acids the development of steep concentration gradients from the serosal to the mucosal surface of the small intestine in hamsters.

The net absorption of glycine and serine in the locust appears to be a relatively slow process, at least under the experimental conditions employed in this investiga-

tion. This may be due perhaps to the relatively large volume of experimental solution introduced into the mid-gut region. It is of some interest in this respect to compare the absorption of amino acids with that of glucose. From the data available (Treherne, 1958*c*) the net absorption of glucose from the caeca at a concentration of 30·0 mM./l. would be expected to be about 54% after an experimental period of 15 min.; the net absorption of serine in a similar period represented only 8·4% of that originally present. The absorption of these amino acids and of glutamine would thus seem to be much less efficient than the facilitated diffusion mechanism postulated for the uptake of monosaccharides in the locust (Treherne, 1958*a*, *b*).

The rapid disappearance of ^{14}C-labelled glycine and serine from the gut lumen was a particularly striking feature in these experiments, indicating the very rapid exchange occurring between the caeca and the haemolymph even though the net absorption was rather slow. These movements were exaggerated in this case as the loss of the ^{14}C-labelled amino acids took place from a small compartment (the caeca) into a relatively large one (the haemolymph and tissues).

Glycine and serine are present at a higher order of concentration than the other amino acids in the haemolymph of the locust. They do not, however, occur in high concentration among the protein amino acids of plants. In the Gramineae, for example, the glycine content is only 2–3% that of arginine and about 4% that of leucine (Lugg, 1941). It seems possible that some of the amino acids released in the mid-gut as the result of the digestion of plant proteins might initially be at a higher concentration than those in the haemolymph, so that some absorption of other amino acids could be achieved as the result of diffusion down a concentration gradient. This process would also tend to be accelerated by the absorption of water from the gut lumen so that it might be expected that other amino acids would be absorbed more rapidly from the food than glycine and serine. The possibility that other amino acids might also be rapidly absorbed by some specific mechanisms has of course not been eliminated.

SUMMARY

1. Chromatographic analysis of the haemolymph revealed the presence of ten amino acids of which glycine and serine occurred in the relatively high concentrations of 33·2 and 34·6 mM./l. respectively. These two amino acids, together with glutamine (10·9 mM./l.), were selected for the study of absorption from the gut lumen.

2. An experimental solution containing ^{14}C-labelled glycine and serine was injected into the gut lumen and the subsequent changes in concentration and radioactivity of the gut fluid were followed.

3. The uptake of ^{14}C-labelled glycine and serine was shown to occur rapidly in the mid-gut region and especially from the lumen of the caeca.

4. The concentrations of glycine and serine, and also of glutamine, in the caecal fluid were found to increase significantly above their concentrations in the haemolymph, an effect which was paralleled by a relatively rapid decrease in fluid volume. During this time rapid exchange of ^{14}C-labelled glycine and serine between the haemolymph and the gut lumen was demonstrated.

5. On the basis of these observations it was concluded that the net absorption of these substances depended, in part at least, upon the diffusion gradient created by the relatively rapid movement of water into the haemolymph.

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THE OSMOTIC AND IONIC REGULATION OF *ASELLUS AQUATICUS* (L.)

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(*Received* 20 *April* 1959)

INTRODUCTION

The isopod, *Asellus*, has been reported from a wide range of conditions in fresh and brackish water. One species, *A. aquaticus*, has a natural salinity range from fresh water (0·15 m-equiv./l. Na; Moon, 1957) to the Baltic (103 mM./l. NaCl; Välikangas, 1933). Thienemann (1913) has also found it occurring in Westphalian brine pools at a concentration of 25·3% salt (equivalent to 434 mM./l. NaCl). A natural salinity range as extensive as this is unusual in freshwater Crustacea. Heuts (1943) has investigated the salinity tolerance of *Asellus* and finds that the highest concentration of diluted sea water in which the animal survives for as long as 24 hr. is equivalent to 300 mM./l. NaCl. This level is considerably below that of the Westphalian brine pools, and the present experiments were undertaken to determine whether the animals could be acclimatized to higher concentrations by gradually raising the concentration of the medium over a long period. Details are also given of the permeability of *Asellus* to salts and water, and the regulation of the concentration of ions in the haemolymph has been studied under various conditions.

MATERIAL AND METHODS

A. aquaticus was collected from a deep drainage ditch on Coe Fen, Cambridge. The concentration of sodium in the water of this ditch varied from 1 to 2 m-equiv./l. over a period of 3 years. In the laboratory the animals were kept either in their natural medium or in tap water, and were fed on alder or willow leaves. They survived well and were apparently normal under these conditions.

Haemolymph collection

Haemolymph samples were obtained from medium to large (8–15 mm.) males by the following procedure. The animal was first dried carefully with filter-paper. A fine Pyrex pipette was then introduced into the rear of the heart by pushing it forward through the intersegmental membrane separating the tergum of the last thoracic segment from the abdomen. Haemolymph flowed readily into the pipette and could be assisted if necessary by gentle suction. 2–5 μl. of haemolymph could be obtained from a single specimen. The haemolymph was blown out of the pipette under the surface of liquid paraffin in a laquered watch-glass. The haemolymph does not clot nor does the osmotic pressure alter appreciably over a period of

48 hr. at room temperature (15–20° C.). However, all determinations of osmotic pressure were carried out as soon as possible after sampling, though in a few cases sodium and chloride were not determined until the following day. Where possible all measurements were made on the haemolymph of a single individual, but in most cases the haemolymph of two or three had to be pooled.

Osmotic pressure

Measurements of the osmotic pressure of both haemolymph and media were made by the micro-cryoscopic method of Ramsay & Brown (1955). Samples were measured in duplicate or triplicate and observations were repeatable to within 0·01° C. Results are expressed in terms of the concentration of NaCl (in mM./l.) which would give the same depression of freezing-point making use of the relationship Δ 1° C. = 285 mM./l. NaCl (Ramsay, 1949). In the range of concentrations used Δ varies directly as the concentration.

Sodium

Sodium concentration was estimated by an E.E.L. flame photometer. Glass micro-pipettes were used to deliver a volume of about 1–2 μl. of haemolymph into 2 ml. of de-ionized water in a Polythene container. The sample was mixed by a stream of air bubbles and the reading given by the photometer was compared with that of a similarly treated volume of a known concentration of NaCl. The standard deviation of a number of similar samples was $\pm 2\%$.

Chloride

Chloride was determined electrometrically by the first method described by Ramsay, Brown & Croghan (1955). The same pipettes were used for chloride as for sodium determinations and again the values obtained for the haemolymph samples were compared with those obtained for the same volume of a known NaCl standard. Results given by a series of similar samples had a standard deviation of $\pm 1\%$.

Heavy water

The proportion of heavy water in haemolymph samples was estimated cryoscopically after microdistillation.

The freezing-point elevation of D_2O and H_2O mixtures was found to vary directly as the concentration of D_2O.

Samples were measured cryoscopically as already described for osmotic-pressure determinations. The sensitivity of the method is less than for density methods, only 1 part in 200 being detectable, but was adequate for the present determinations.

RESULTS

Salinity tolerance

Animals were placed in NaCl solutions of various concentrations for 7 days. The greatest concentration which an individual could survive for this period ranged from 200 to 270 mM./l. Higher concentrations could be tolerated for shorter times.

Since these concentrations do not approach those at which Thienemann (1913) found *Asellus* occurring naturally, the concentration was increased slowly over a period of several months to see if the animals could be acclimatized to higher salinities.

The animals were placed in diluted sea water (osmotic pressure equivalent to 34 mM./l. NaCl) and provided with food. The concentration of the medium was slowly raised by evaporation and the addition of concentrated sea water. No animals survived a concentration greater than 220 mM./l. A repetition of this experiment gave similar results, the last of the experimental animals dying when the concentration reached 200 mM./l. Many of the young released from the brood pouch of a female during the course of this experiment survived, however, until the concentration reached 250 mM./l.

The normal haemolymph concentration

Determinations were made of the osmotic pressure (O.P._i) of the haemolymph of both freshly caught animals and animals which had been kept in Coe Fen water under as natural conditions as possible in the laboratory. The mean value of O.P._i obtained was 150·5 mM./l. NaCl ($n = 121$). Most of the determinations were made in the months September–May inclusive. This figure may be compared with other mean values for O.P._i of *Asellus* given by Parry (1953) and Widmann (1935) of 141 and 251 mM./l. NaCl respectively. Heuts (1943) has criticized Widmann's results and his own values for O.P._i are about half those given by Widmann.

O.P._i has also been determined over the salinity range tolerated in nature by *Asellus* to determine to what extent the haemolymph concentration is regulated. Excluding the special case of its occurrence in the Westphalian brine pools, the natural salinity range is 0·15 m-equiv./l. Na to 103 mM./l. NaCl. O.P._i near the lower limit of this range was determined by washing the animals with distilled water for a period sufficient to decrease the haemolymph concentration markedly (to 65% in 48 hr.), and then allowing them to take up salts from dilute solutions of NaCl for a period of 7 days. Table 1 shows that O.P._i is close to the normal freshwater value in a medium with a concentration of 0·06 mM./l. NaCl and at the normal value in solutions containing more than 0·088 mM./l. NaCl. This latter concentration is well below the minimum natural lower limit of 0·15 mM./l., at which therefore a haemolymph concentration of 150 mM./l. is to be expected. O.P._i at 103 mM./l. was determined after acclimatizing animals to this concentration of NaCl for 7 days. After this time O.P._i was close to 170 mM./l.

The rise in the haemolymph concentration over the normal 700-fold range of medium concentration is thus only from 150 to 170 mM./l. or some 15%. It seems clear that the active uptake mechanisms display very considerable sensitivity in regulating the concentration of the haemolymph.

The haemolymph concentration in hypertonic media

The relationship between O.P._i and the NaCl concentration of the medium was investigated during acclimatization of the animals to saline solutions.

Table 1. O.P.$_i$ *and* Na$_i$ *at low medium concentrations*

	Controls		Animals in distilled water for 48 hr. then 7 days in dilute NaCl solutions		
	Medium		Medium concentration (mM./l. NaCl)		
	Fresh water	48 hr. Distilled water	0·06	0·088	0·6
O.P.$_i$ (mM./l. NaCl)	150·5 ± 8 n = 121	103 ± 10 n = 5	134 144 150	145 150 154	154 153 151
Na (m-equiv./l.)	137 ± 8 n = 60	90 Pooled haemolymph of above five animals	119	125	127
			Pooled haemolymph of above animals		

For short-term acclimatizations animals were placed in NaCl solutions of different concentrations for 6–8 days and the haemolymph then sampled. (It will be shown in a later section that the haemolymph concentration reaches equilibrium in hypertonic media within 24–48 hr.) O.P.$_i$ rose as the medium concentration was increased. It approached isotonicity with the medium at high concentrations but was still hypertonic at the highest medium concentration compatible with life (Fig. 1). Heuts (1943) obtained similar results.

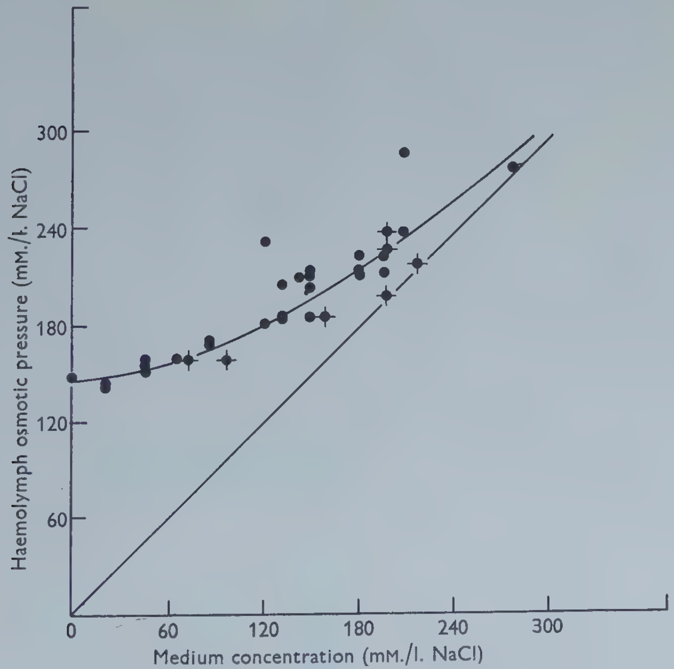

Fig. 1. The osmotic pressure of the haemolymph of animals acclimatized for short and long periods to concentrated saline. ●, Acclimatization period 6–8 days; ✦, acclimatization period 1–4 months. The diagonal line represents isotonicity.

During the long-term salinity acclimatization experiments, described above, animals were removed at intervals and their haemolymph sampled. These animals had a very slightly lower O.P.$_i$ at any given medium concentration than animals only 1 week in media of the same concentration (Fig. 1).

The composition of the haemolymph

The concentrations of three of the major ionic constituents of the haemolymph are compared in relation to the total osmotic pressure of normal animals in Table 2. Sodium and potassium together account for some 96% of the O.P.$_i$ due to cations. Calcium and magnesium have not been determined, but by analogy with other freshwater Crustacea such as *Astacus* may be expected to make up a considerable proportion of the remaining 4%. The un-ionized organic contribution to O.P.$_i$ must therefore be small.

As has been mentioned, it is possible to vary the animal's total haemolymph concentration by placing it in distilled water or various concentrations of saline. The relationship between Na_i, Cl_i and O.P.$_i$ over the viable range is summarized in Fig. 2. The figure shows that over the whole range most of O.P.$_i$ is due to sodium and chloride. As would be expected if there is a proportion of large organic anions in the haemolymph which are not readily lost from the body, the Cl_i/O.P.$_i$ ratio shows some slight fall at low values of O.P.$_i$. The ratio Na_i/O.P.$_i$ remains constant (Table 3).

Table 2. *The normal osmotic pressure, sodium, chloride and potassium concentrations of the haemolymph*

	Mean	Range	Number	σ
O.P.$_i$	150·5	136–171	121	±8
Na_i	137	120–159	60	±8
Cl_i	125	97–145	33	±7
K_i	7·4	6–8·5	5	±0·4
Na_i/O.P.$_i$ %	91	—	—	—
K_i/O.P.$_i$ %	4·9	—	—	—
Cl_i/O.P.$_i$ %	83	—	—	—

O.P.$_i$ values expressed as mM./l. NaCl. σ = standard deviation. Na_i, Cl_i, K_i as m-equiv./l.

Table 3. *The ratios of* Na_i *and* Cl_i *to osmotic pressure over a range of total haemolymph concentration*

O.P.$_i$	Na_i/O.P.$_i$ (%)	No.	σ (%)	Cl_i/O.P.$_i$ (%)	No.	σ (%)
< 103	89·7	7	±5·4	77·4	15	±11·6
103–120	88·7	7	±4·2	82·1	7	±4·2
120–137	88·5	15	±3·3	83·9	8	±6·5
137–154	90·0	44	±3·0	84·3	25	±3·8
154–171	89·3	24	±2·5	85·0	13	±5·9
171–188	90·0	7	±3·4	87·0	1	± —
> 188	91·0	13	±3·4	86·9	6	±6·4

O.P.$_i$ in mM./l. NaCl.

Permeability to water

Large animals were placed in a 50% solution of D_2O at 18° C. After 25 min. samples of haemolymph were taken and the percentage of D_2O was determined in three individuals. The percentage of haemolymph water exchanged in this time had a mean value of 53%. This value is intermediate between some other comparable values obtained on Crustacea. Ussing (in Krogh, 1939) found an 80% exchange within 2 min. for *Daphnia* and approximately 25% exchange in 1½ hr. for *Artemia*. It is difficult to interpret D_2O exchange experiments in terms of net rate of water entry, but the fact that it is possible for D_2O to exchange to this extent with the haemolymph water in so short a time indicates that some portion of the body is permeable to water. Osmotic uptake of water may therefore be expected to occur when the animal is in its normal fresh-water environment. This water is presumably excreted as urine.

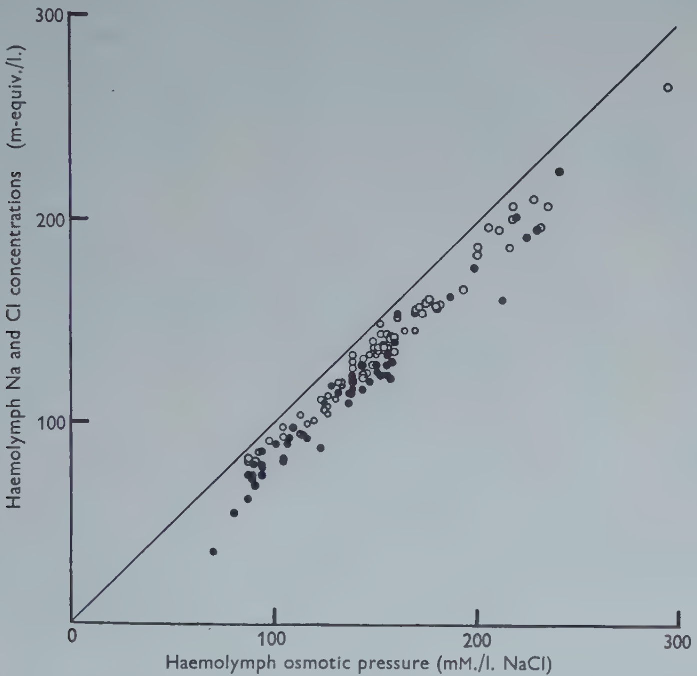

Fig. 2. The ionic constitution of the haemolymph over the viable range of osmotic pressure. O, Na concentration; ●, Cl concentration. The diagonal line represents osmotic pressure of the haemolymph.

Permeability to ions

The inward penetration of sodium into the body was shown by determining O.P.$_i$ and Na$_i$ of animals placed in a solution of 1% NaCl. The temperature was 15–18° C. O.P.$_i$ and Na$_i$ rose rapidly in the first few hours, the haemolymph regaining hypertonicity to the medium with respect to sodium after about 8 hr. Na$_i$ continued to rise, however, until after 24 hr. it had reached a value of 197 m-equiv./l.

at which it remained constant (Fig. 3). The concentration of the medium was checked at intervals and did not vary. Heuts (1943) found that hypertonicity with respect to chloride is regained 6 hr. after *Asellus* is placed in a sea-water medium equivalent to 222 mM./l. NaCl. Osmotic withdrawal of water might account for the rise in ion content of the haemolymph during the first few hours before O.P.$_i$ becomes hypertonic to the medium. Active movement of ions against the concentration gradient must account for the further increase in haemolymph ion concentration after this time.

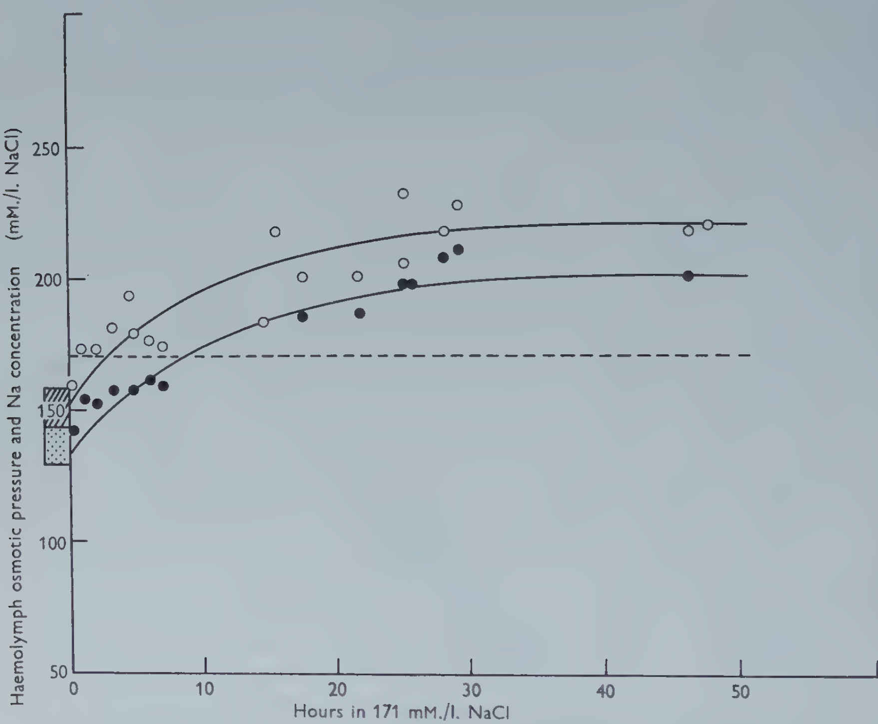

Fig. 3. The time course of rise of O.P.$_i$ and Na$_i$ of animals placed in hypertonic NaCl. ●, Na$_i$; ○, O.P.$_i$. --- Medium concentration. Hatched box: mean O.P.$_i$ ± standard deviation of normal animals. Dotted box: mean Na$_i$ ± standard deviation of normal animals.

Animals exposed to continuously flowing de-ionized water lose ions to the medium, sodium being lost at a rate sufficient to lower O.P.$_i$ and Na$_i$ to about 70% of normal in 24 hr. The further responses to a flow of de-ionized water are complex and will be discussed in detail elsewhere.

The haemolymph concentration during starvation

The permeability of the body to ions and water and the fairly rapid rate of salt loss in de-ionized water suggest that the hypertonicity of the haemolymph in fresh water must be maintained either by a high salt intake in the food or by active up-

take. Krogh (1939) has shown that starved *Branchipus* and *Apus* lose chloride to their medium. Pannikar (1941) has noted a similar effect of *Chirocephalus*, and Heuts claims that chloride is lost from starved *Asellus*.

The effect of starvation on *Asellus* has been re-investigated. Animals taken from their normal habitat were placed in filtered water at 10° C. Haemolymph samples were taken at intervals of 24 hr. for a total period of 8 days. For each determination the haemolymph of 2 or 3 animals was pooled and O.P.$_i$, Na_i and Cl_i measured. No significant change occurred in any of these factors (Fig. 4).

If sodium is normally lost from the animal at a rate similar to that initially observed in de-ionized water then, during an 8-day period, almost the whole of the sodium in the body should have been lost. In fact O.P.$_i$, Na_i and Cl_i are observed to remain at their normal levels, within the range of individual variation. It seems clear that the loss of ions from the body is balanced by a simultaneous active uptake of ions from the medium. The maintenance of Na_i and Cl_i in *Asellus* is therefore independent of a supply of sodium and chloride in the food.

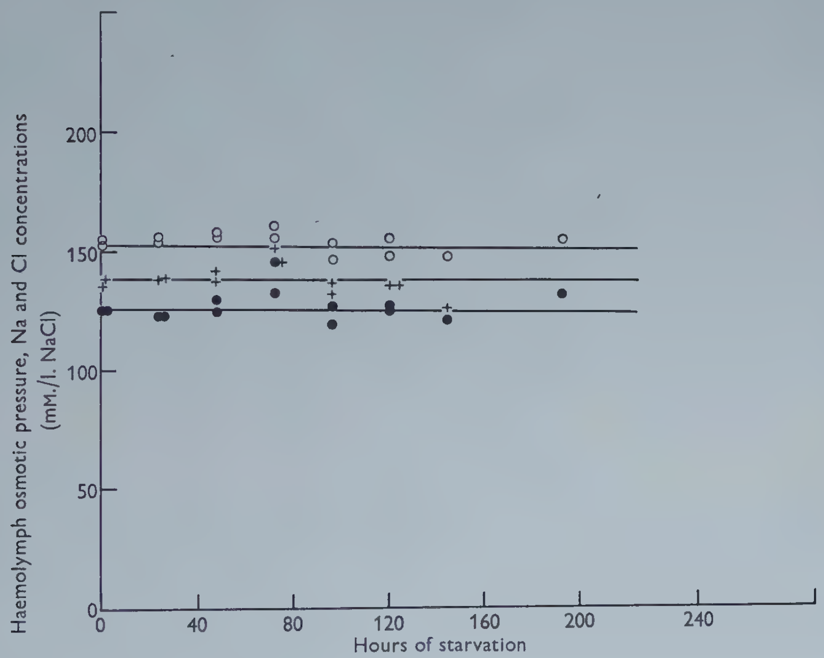

Fig. 4. The haemolymph osmotic pressure, Na concentration and Cl concentration during starvation. ○, O.P.$_i$; +, Na_i; ●, Cl_i.

DISCUSSION

The osmotic and ionic concentrations of the haemolymph of *A. aquaticus* are typical of those of other freshwater Crustacea.

Although *Asellus* tolerates a rise in the salt concentration of its medium up to values approximately the same as the normal haemolymph concentration, it cannot

be regarded as being an animal well adapted to life at high salinities. Long-term acclimatization to a gradually increasing concentration has little effect in increasing the salinity tolerance. It thus differs from the only other freshwater isopods previously investigated. Both *Caecospheroma virei* (Derouet, 1952) and *Mesidotea entomon vetterensis* (Lockwood & Croghan, 1957) have been found to tolerate all salinities from fresh water to sea water (at least for the duration of the experiments) and these must therefore be regarded as being more truly euryhaline species than *Asellus aquaticus*. The occurrence of the last species in the highly saline Westphalian brine lakes (Thienemann, 1913) cannot be accounted for in terms of the salinity tolerance of other populations and is possibly to be regarded as the result of genetic selection over a long period.

The maintenance of Na_i against a concentration gradient of approximately 100:1 must result from the replacement of ions from the medium at the same rate as they are lost from the body by diffusion and in urine. The continued maintenance of O.P.$_i$, Na_i and Cl_i during 8 days of starvation shows that *Asellus* can, if necessary, replace NaCl lost from the body solely by active uptake from the medium. The intake of these ions is thus not dependent on the food supply.

Attention has been drawn to the inability of *Asellus* to tolerate high salinities. It may be noted, however, that the effective range of concentration tolerated by *Asellus* is considerable. Excluding the occurrence in the Westphalian brine lakes, the animal is known to occur over a 700-fold range of medium concentration. This range of concentration is 6 times the range tolerated by the highly euryhaline brine shrimp *Artemia* under experimental conditions (Croghan, 1958), though it is true that the osmotic problem to be overcome by the latter is very different. The sensitivity of the ion-regulating mechanisms of *Asellus* is shown by the fact that, under experimental conditions, the haemolymph concentration remains practically constant over approximately the natural range. O.P.$_i$ is only some 15% higher in media of 100 mM./l. than it is in media of 0·150 mM./l. NaCl.

SUMMARY

1. The freshwater isopod *Asellus aquaticus* can be adapted to media ranging in concentration from 0·088 mM./l. NaCl to 200 mM./l. NaCl.

2. In fresh water O.P.$_i$ has a mean value of 150·5 mM./l. NaCl. In more concentrated media O.P.$_i$ approaches isotonicity with the medium.

3. The mean ion concentrations of the haemolymph of animals in fresh water are Na_i = 137 m-equiv./l., Cl_i = 125 m-equiv./l. and K_i = 7·4 m-equiv./l.

4. Na and Cl account for most of O.P.$_i$ throughout the viable range of haemolymph concentration.

5. Evidence is presented which suggests that the animal is fairly permeable to salts and water.

6. During 8 days' starvation there is no change in O.P.$_i$, Na_i or Cl_i. Sodium and chloride lost from the body can therefore be replaced by active uptake in the absence of food.

I would like to thank Dr J. A. Ramsay, F.R.S., for all his interest and advice and also the Department of Scientific and Industrial Research for a maintenance grant. I am also indebted to Dr P. King who kindly translated the paper by M. J. Heuts.

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THE REGULATION OF THE INTERNAL SODIUM CONCENTRATION OF *ASELLUS AQUATICUS* IN THE ABSENCE OF SODIUM CHLORIDE IN THE MEDIUM

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(*Received* 20 *April* 1959)

INTRODUCTION

Many small freshwater animals survive in distilled water for a considerable time. The factors concerned in the maintenance of the ion concentrations in the haemolymph at levels compatible with life under these circumstances have not been fully examined.

Beadle & Cragg (1940), measuring the haemolymph concentration of *Gammarus pulex* which had been in distilled water for various periods, showed that there is initially a fairly rapid fall in the haemolymph chloride concentration followed by a long period during which the concentration falls very slowly. They have therefore suggested that retention of ions may play an important part in the maintenance of the ion concentrations in the haemolymph.

In the present paper details are given of the effect of treatment with distilled water on the haemolymph concentration of the isopod *Asellus aquaticus*. In addition, a study has been made of the actual rate of loss of sodium from the body of this animal to demonstrate that the maintenance of an almost constant haemolymph concentration does not necessarily imply retention of ions.

MATERIALS AND METHODS

The *Asellus* used were in general large males, though a few unberried females were also included. Specimens were from a drainage ditch on Coe Fen, Cambridge. The animals were not fed during the course of the experiments. It has been shown that a starvation period of 8 days does not affect the ionic or total concentration of the haemolymph (Lockwood, 1959).

Osmotic pressure was estimated by the method of Ramsay & Brown (1955), sodium by flame photometry and chloride by the first method of Ramsay, Brown & Croghan (1955). For details of procedure and method of haemolymph collection see Lockwood (1959).

In the earlier experiments on the effect of ion lack on the haemolymph concentration the animals were kept in a 700 ml. chamber through which a slow current of distilled water was passed. The purity of the water in the chamber was tested at intervals and the sodium concentration was not found to rise above 4 μequiv./l. In

later experiments smaller chambers of 3–10 ml. were used and the incoming stream of water (5–25 ml./min.) was first passed through a column of Amberlite M.B. 3 ion-exchange resin. The de-ionized water prepared in this way did not contain more than 1 μequiv./l. sodium.

Animals used in tracer experiments were also kept in the 3–10 ml. chambers which were mounted on a tray designed to fit into an E.R.D. lead castle. The activity of tracer in the animals could thus be readily determined. The water was de-ionized with resin and lifted by an air pump to a Polythene storage tank. Continuous aeration and circulation were thus achieved. All the tubing used was Polythene.

The fact that the animal has freedom of movement in the chamber entails the disadvantage that the thickness of water between the animal and the counter is liable to vary from time to time. This results in a variable degree of absorption of β-particles by the water. Errors arising from this source were reduced by interposing a brass screen (990 mg./cm.^{2}) between the chamber and counter. This screen absorbs all the soft β-particles but allows a high and constant proportion of the γ-rays to pass. Absorption of γ-rays is not appreciably altered by the small changes in water thickness.

A standard was counted before and after the animal to correct the count rate and decay, and in addition the usual corrections for background and dead time were applied.

All experiments were carried out at room temperature (15–22° C.). In the first series of tracer experiments animals were loaded in a solution containing ^{22}Na until their tracer count reached a steady state. This took about 3 weeks and food was provided during the loading period. In a later series of experiments animals were loaded for only a few hours before being washed out with de-ionized water.

RESULTS

The haemolymph concentration in distilled water

Most animals survived in distilled water for 6–8 days, the longest observed period of survival being 16 days. Osmotic pressure (O.P.$_i$) falls rapidly during the first day, but after this time only a very slow decrease in the concentration occurs (Fig. 1). This appears to suggest that the rate of salt loss has been much diminished as the ratio Na/O.P. remains constant (Lockwood, 1959). Less haemolymph can be obtained from animals which have been washed out for a few hours than from normal controls of a similar size.

The initial rate of fall of Na_i is about 1·6% Na_i/hr. This is probably close to the rate of loss by diffusion and in the urine in fresh water, since the diffusion gradient is little altered by transferring the animals to distilled water. As Na_i decreases so does the concentration gradient between haemolymph and medium. This would be expected to result in a gradual rather than a rapid decrease in the loss of salt from the body by diffusion. However, after the first day in distilled water Na_i falls only very slowly.

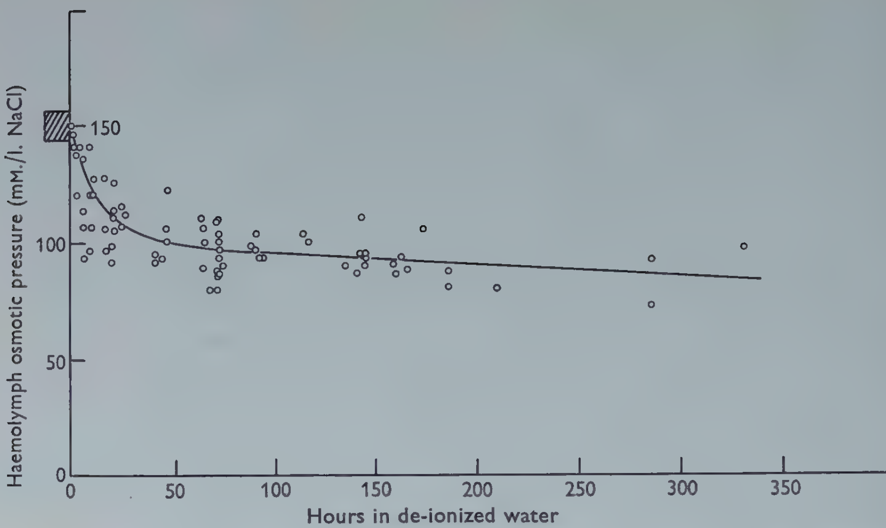

Fig. 1. The haemolymph osmotic pressure after various periods in de-ionized water. The hatched area represents the mean haemolymph osmotic pressure ± the standard deviation of normal animals.

The loss of ^{22}Na *in distilled water*

The possibility that a true measure of the rate of salt loss from the body is not obtained by measurements of the haemolymph concentration was therefore tested by measuring the rate of loss of ^{22}Na from loaded animals. After being loaded to a steady state the animals were placed in small plastic chambers and washed with de-ionized water. The count rate was recorded at intervals. The curve of the loss of ^{22}Na from the body differed in shape from that of the Na_i. The initial rapid fall and levelling out were now replaced by a slower fall, and only a comparatively slight decrease in the rate of loss with time (Fig. 2). Four repetitions of the experiment, though differing quantitatively, confirmed this difference.

Loss of ^{22}Na is proportional to rate of sodium loss from the body. It seems, therefore, that sodium is lost at a rate which only declines with time to about a quarter or half the initial value. Clearly sodium is not being retained in the body to the extent that a study of the concentration of ions in the haemolymph might appear to indicate.

In a number of cases animals were loaded in ^{22}Na for only a few hours before being washed out. The curve of falling ^{22}Na obtained from these animals differed in a number of respects from that of the long-term loaded animals. The ^{22}Na falls rapidly to about 40–50% of its initial value, and then only a very slow loss occurs for a long period (Fig. 3). In some cases the loss may again increase slightly before the death of the animal. Determinations of the $O.P._i$ and Na_i of animals which had lost 50–60% of their initial ^{22}Na showed that the haemolymph concentration was still 60–65% of normal.

If all the sodium in the body of *Asellus* were in the haemolymph, then the length of the initial loading period in tracer could not result in any qualitative difference

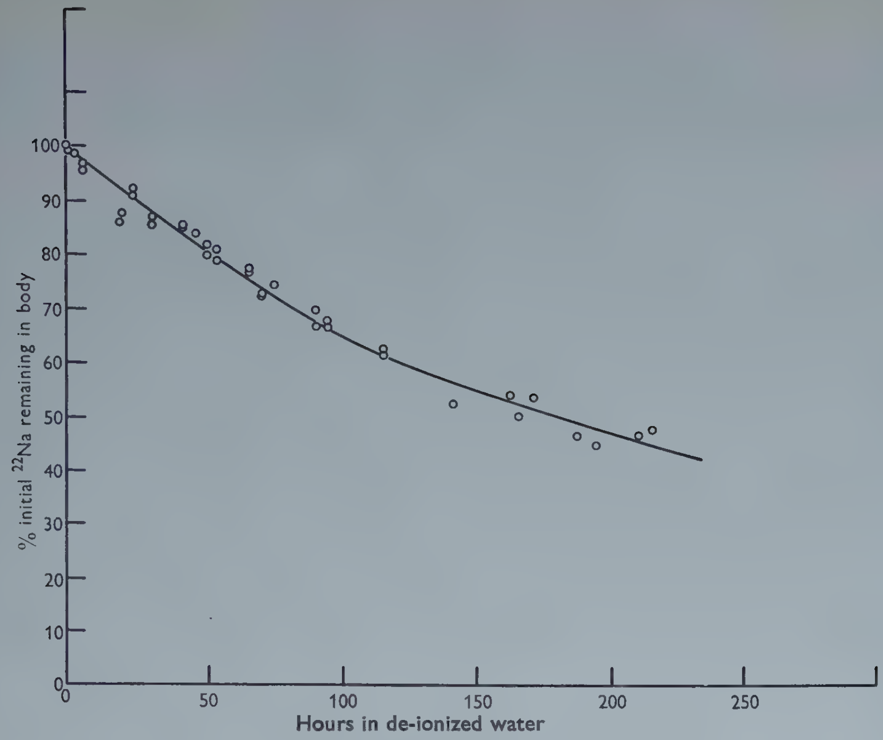

Fig. 2. The falling ^{22}Na count of an animal loaded to steady state and then washed in de-ionized water.

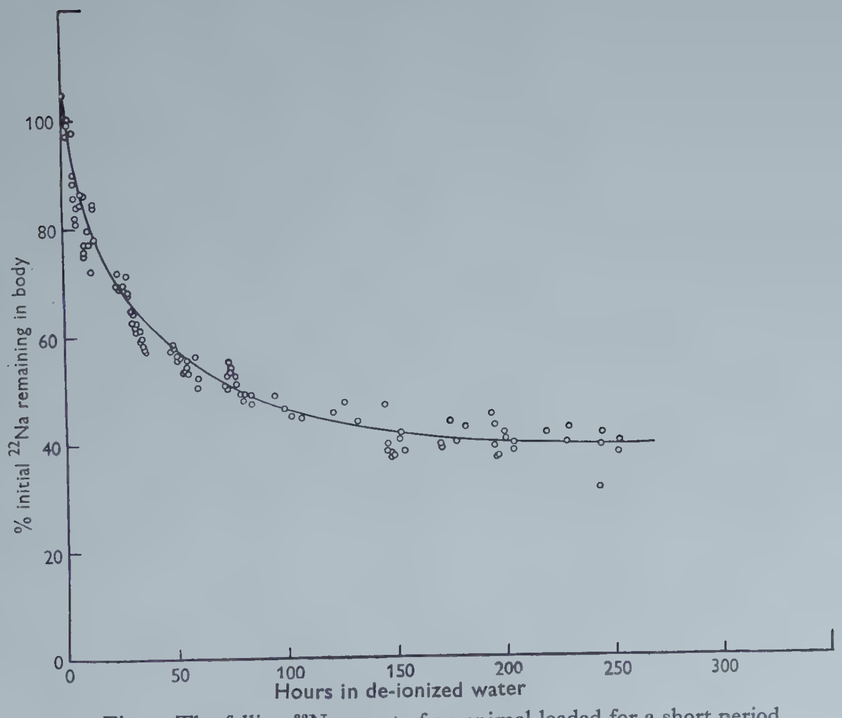

Fig. 3. The falling ^{22}Na count of an animal loaded for a short period and then washed in de-ionized water.

in the curves of loss of ^{22}Na on washing with de-ionized water. These results are therefore incompatible with the assumption that all the sodium in the body of *Asellus* is in the haemolymph.

DISCUSSION

Beadle & Cragg (1940) have postulated that retention of ions is an important feature of the survival of such animals as *Gammarus pulex* in diluted media. Like *Gammarus*, *Asellus* has been found to maintain a remarkably constant haemolymph concentration (after an initial rapid fall) for a long period in running distilled water. However, the fact that the rate of loss of ^{22}Na from long-term loaded animals in distilled water is only decreased slightly with time indicates that the constancy of the Na_i does not give a true picture of the actual loss of sodium from the animal. In *Asellus*, at least, a decreased rate of loss of ions is not primarily responsible for the maintenance of the Na_i when the animal is in distilled water.

Two possible theories could explain the apparent anomaly between loss of sodium and maintenance of Na_i. Either (*a*) sodium from the tissues replaces that lost from the haemolymph and thus maintains Na_i, or (*b*) the volume of the haemolymph is decreased as the concentration falls so that the concentration is maintained at a higher level than would be expected from the amount of sodium lost from the body.

As the short-term and long-term loaded animals give curves of different shapes on washing out, it appears that a considerable proportion of the total sodium in the body is not rapidly exchangeable and hence presumably is not in the haemolymph. There is as yet no evidence to suggest that this extra-haemolymph sodium may be released to assist in the maintenance of the haemolymph concentration.

Attempts to determine changes in the haemolymph volume directly have not been successful as the total amount of haemolymph in *Asellus* is small. The observation, however, that it becomes difficult to obtain normal quantities of haemolymph from washed-out animals, though qualitative, may indicate that there is some reduction of the haemolymph volume as the concentration falls.

Camien, Sarlet, Duchâteau & Florkin (1951) have shown that a considerable part of the osmotically active substances in the cells of Crustacea are organic in nature. Any reduction in the haemolymph concentration would therefore be expected to result in a movement of water into the cells. Shaw (1955) has shown that the muscle cells of *Carcinus* swell as the result of just such a water shift when the haemolymph concentration is lowered. It is thus not unreasonable to suppose that a similar water shift to the cells occurs in the case of *Asellus*. If the cells are regarded as being always isotonic with the haemolymph, the decrease of haemolymph volume would be sufficient to account for the maintenance of haemolymph-ion concentration.

Active processes may possibly play some part in the slight decrease in the rate of loss of sodium from the body, but unfortunately no details are available as to the effect of a reduction in the haemolymph concentration on the output and concentration of the urine.

The presence of a considerable quantity of sodium in the tissues is an unusual feature of *Asellus*. This subject will be dealt with in detail in a later paper and will not be considered further here.

SUMMARY

1. The freshwater isopod *Asellus aquaticus* has been observed to survive in continuously flowing de-ionized water for up to 16 days.

2. The haemolymph osmotic pressure, and the concentrations of sodium and chloride fall rapidly to about 65% of the normal value and then decrease very slowly until death.

3. Animals loaded to a steady state in ^{22}Na and then washed with de-ionized water lose tracer at a rate which only decreases slightly between the beginning of washing out and the death of the animal.

4. It is suggested that a movement of water from haemolymph to cells as the haemolymph concentration falls may partially account for the maintenance of the haemolymph concentration despite the steady ion loss.

5. Animals loaded for only a short period in tracer before washing out lose about 50–60% of their ^{22}Na after a few hours in de-ionized water and then show little further loss for a long period.

6. The differences in the tracer loss curves following long-term and short-term loading have been interpreted as indicating that a considerable proportion of the total sodium of *Asellus* is not in the haemolymph.

I would like to thank Dr J. A. Ramsay, F.R.S., for all his interest and advice in this work, and Dr P. C. Croghan for many helpful discussions. I am indebted to the Department of Scientific and Industrial Research for a maintenance grant and the Earl of Moray Endowment for the Promotion of Original Research for a grant for instruments.

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THE EXTRA-HAEMOLYMPH SODIUM OF *ASELLUS AQUATICUS* (L.)

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(*Received* 20 *April* 1959)

(With Plate 11)

INTRODUCTION

In an earlier paper Lockwood (1959*b*) has suggested that a considerable proportion of the total body sodium of *Asellus aquaticus* is not in the haemolymph. Camien, Sarlet, Duchâteau & Florkin (1951) have found that a large proportion of the osmotic activity of crustacean tissue is due to organic components, and direct determination of the ionic concentration of *Carcinus* muscle fibres (Shaw, 1955) has shown that the sodium concentration of the muscle is only about one-ninth that of the haemolymph. The presence of a considerable quantity of sodium in the tissues of *Asellus* is therefore unusual. The purpose of the experiments described in this paper was to determine the quantity and location of this extra-haemolymph sodium.

MATERIALS AND METHODS

The *Asellus* used were in general large males, though a few unberried females were also included. Specimens were from a drainage ditch on Coe Fen, Cambridge.

Sodium concentration was determined by flame photometry and chloride by the first method of Ramsay, Brown & Croghan (1955). For details of procedure and method of haemolymph collection see Lockwood (1959*a*).

The total sodium and chloride content of the body were estimated as follows. Batches of 5–10 animals were blotted dry and weighed. (By repeated weighings of an individual it had been established that live animals could be weighed to an accuracy of $\pm 2\cdot 5\%$.) After weighing, the animals were killed by immersion in acetone and dried to constant weight at 100–105° C. The total body water was thus obtained. The dried bodies were then ashed in an electric furnace at 450–500° C. for 3 hr. The resultant ash was taken up in 2 ml. of N/5 H_2SO_4. This procedure was tested on a known standard of NaCl and did not result in any loss. Aliquots of the dissolved ash were compared with NaCl standards to determine the sodium and chloride content. Haemolymph samples were collected from animals similar to those ashed.

The location of sodium in the animal was ascertained by a combination of freeze-drying and autoradiographic techniques. Animals were loaded in a ^{22}Na solution

for at least 3 weeks. (This time is sufficient to ensure that all the sodium in the body is in equilibrium as regards its $^{22}Na/^{23}Na$ ratio.) After rinsing in distilled water the animals were dropped into isopentane cooled with liquid nitrogen. When the isopentane had solidified, the block containing the animal was decanted into a holder in the head of an Edwards Vacuum freeze-dryer. The head was evacuated and the temperature allowed to rise to -40 to $-35°$ C. at which it was maintained for 4 days. The dried animals were embedded in wax and sectioned at 20 μ. Sections were mounted on slides and squares of Kodak Autoradiographic film were wrapped round each slide with the 10 μ gelatine backing between the sections and the emulsion. The dry film was pressed gently into the wax to ensure close contact between film and section. It is believed that this technique minimized dispersion of sodium from areas of local concentration existing in life. After 20–31 days exposure the films were developed and the slides dewaxed and stained.

RESULTS

Ion spaces

If all the ions of a given species are in the haemolymph then a measure of the ion space will approximate to the haemolymph volume:

$$\text{Ion space} = \frac{\text{concentration of ion in total body water} \times 100}{\text{concentration of ion in the haemolymph}}.$$

Assuming that all the chloride is in the haemolymph then any excess of the corresponding sodium space over the chloride space will represent the percentage of the total sodium which is in the tissues. The small but unknown chloride content of the cells cannot be taken into account so the value obtained will represent the minimum percentage of the total sodium in the tissues.

The sodium and chloride spaces were determined and the results are given in Table 1. In general, different batches of animals were used to determine sodium and chloride spaces, but where both determinations were made on the same batch the 20–30% difference was also observed.

Table 1. *The sodium and chloride spaces of* Asellus

	Mean	σ	No. of determinations
Chloride space	53%	±10%	14
Sodium space	80%	±10%	18

Values as percentage total body water. σ = standard deviation.

Uniform distribution throughout the tissues of 20–30% of the total body sodium would require the cellular sodium concentration to have an unusually high level. It was therefore considered that some of the sodium might be concentrated in restricted areas. Such areas were located by the freeze-drying and autoradiographic technique.

Autoradiographic results

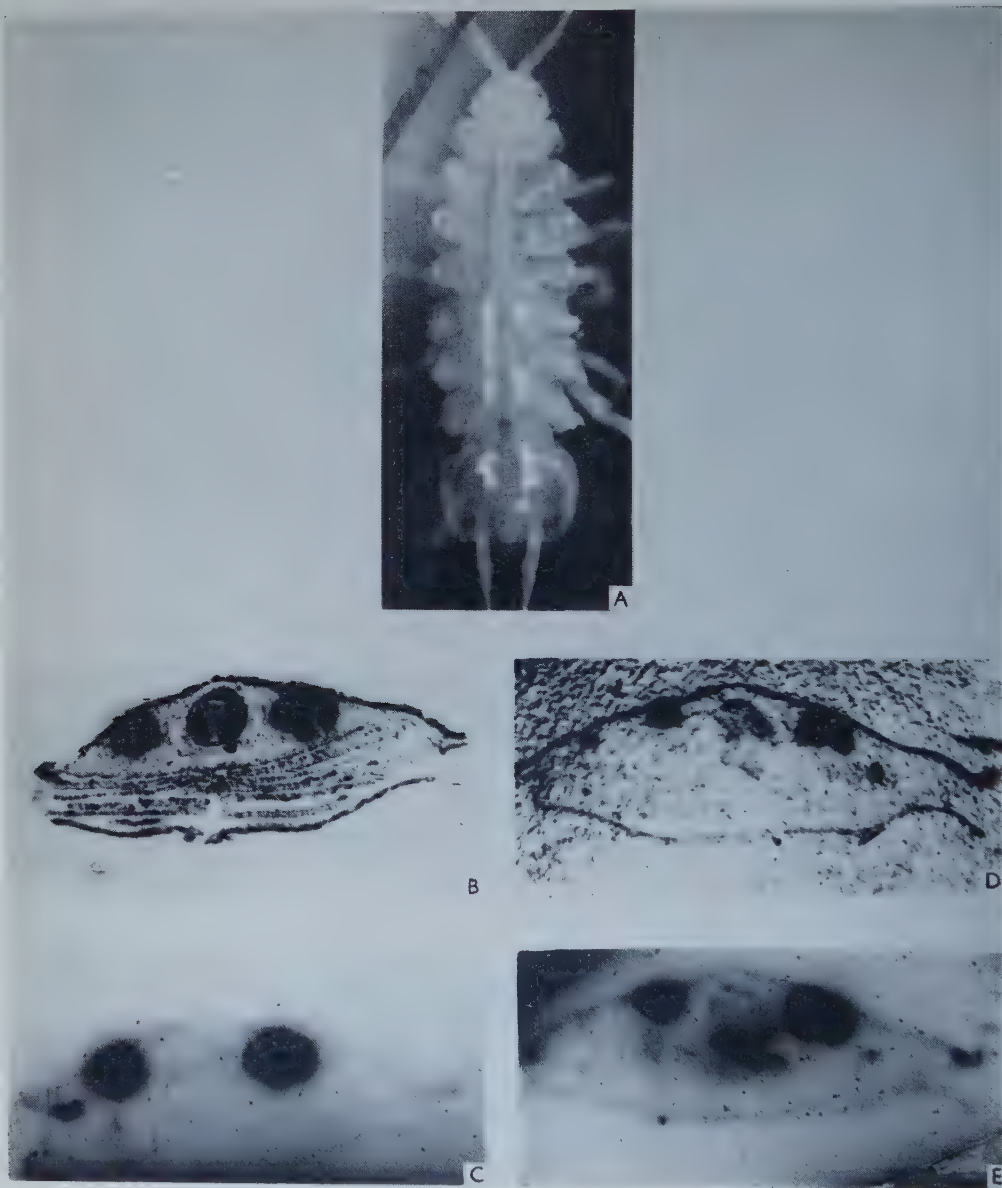
Films covering sections taken from the experimental animals gave clear autoradiographs, whilst those over sections from unloaded control animals were blank.

The autoradiographs (Pl. 11, C, E) show that there is a general diffuse blackening over the whole section, presumably caused by ^{22}Na in the haemolymph. The cuticle is somewhat more clearly defined, but by far the most intensely darkened portion of the film is that overlying certain regions lateral to the gut in the abdomen and posterior part of the thorax. The four 'digestive' tubules and the ovaries also give rise to some blackening. The degree of darkening of the film is proportional to the radiation it receives and hence to the concentration of tracer beneath it. Therefore most of the extra-haemolymph sodium is presumed to be concentrated in these regions. Microscopic examinations of the regions lateral to the gut in the sections (Pl. 11, B, D) show them to be large single cells packed with a fine crystalline material. In an intact pale animal these cells (which constitute the organ of Zenker) are opaque and can be seen clearly with the naked eye (Pl. 11, A). The distribution of the cells differs in *Asellus aquaticus* and *A. meridianus* (see Appendix).

Dresel & Moyle (1950) found that the body of *Asellus* contained a high proportion of uric acid (6·4 mg./g. wet weight). This represents some 3% of the dry weight of the animal, a considerable quantity to be stored in a freshwater animal which is freely permeable to water. Dresel & Moyle suggest that the cells of Zenker's organ might be the site of the uric acid and this has now been confirmed. In *A. aquaticus* the cells of Zenker's organ are confined to the rear half of the animal and only this half gives a positive test for uric acid when ground up in Benedict's reagent and Na_2CO_3. Similar treatment of the crystalline contents of individual cells gave a positive test for uric acid. It appears therefore that the content of these cells is in part made up of crystalline sodium urate.

DISCUSSION

The presence of some 20–30% of the total sodium of *Asellus* in the tissues suggested that the sodium concentration of the cells of this animal might be unusually high. It has now been shown, however, that the extra-haemolymph sodium is not generally distributed within the body, but that a considerable proportion is concentrated in the cells of Zenker's organ on either side of the gut. Lesser quantities are present in the digestive tubules, ovaries and cuticle. There are therefore no grounds to suppose that the general body cells have sodium content which is in any way unusual. The cells of Zenker's organ give a positive test for uric acid, and the quantity of uric acid in the body, if all in the form of mono-sodium urate, would be sufficient to account for as much as 40% of the total body sodium. There is as yet no evidence as to the function of the sodium in the cells of Zenker's organ, and it would be interesting to know if sodium is associated with uric acid in other cases where this nitrogenous waste product is stored intracellularly.



LOCKWOOD—THE EXTRA-HAEMOLYMPH SODIUM OF *ASELLUS AQUATICUS* (L.)

(*Facing p.* 565)

SUMMARY

1. Some 20–30% of the total body sodium of the freshwater isopod *Asellus aquaticus* is not in the haemolymph.

2. Autoradiography shows that most of this sodium is not uniformly distributed in the tissues but is localized in the cells of Zenker's organ with lesser amounts in the ovaries, digestive tubules and cuticle.

3. Uric acid is also present in the cells of Zenker's organ.

4. No evidence is yet available to suggest the function of the sodium in the cells of Zenker's organ.

I would like to thank Dr J. A. Ramsay, F.R.S., for his interest and advice in this work, Dr E. Robson for assistance with the freeze-drying, and the Department of Scientific and Industrial Research for a maintenance grant.

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APPENDIX

The two common British species of *Asellus*, *aquaticus* and *meridianus*, are very similar in general appearance. However, differences in the distribution of the urate cells which make up Zenker's organ may readily be seen. From the ventral side these cells are visible as white or yellowish blotches in the abdominal region. Whilst *A. meridianus* has a crescentic band of urate underlying the rectum, *A. aquaticus* has the cells widely separated on either side of the rectum. In addition, in the paler animals it can be seen that *A. meridianus* has uric cells in all the thoracic segments, whilst in *A. aquaticus* they are confined to the last three thoracic segments.

EXPLANATION OF PLATE

A. Unpigmented *A. aquaticus* displaying urate-containing cells (white blobs) lateral to the gut in the abdomen and posterior thorax.

B. Transverse section of abdomen showing pleopods (ventral), gut (mid-dorsal) and two groups of urate cells (latero-dorsal).

C. ^{22}Na autoradiograph of B. Demonstrates presence of sodium in urate cells.

D. Transverse section of abdomen showing gut, urate cells and digestive tubules (ventral to gut).

E. ^{22}Na autoradiograph of D. Blackening is produced by urate cells and digestive tubules.

NITROGEN EXCRETION IN NYMPHS OF *AESHNA CYANEA* (MÜLL.) (ODONATA, ANISOPTERA)

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(*Received* 7 *May* 1959)

INTRODUCTION

The present paper reports the results of a brief study on the excretion of ammonia and uric acid in nymphs of *Aeshna cyanea*. It is well known that terrestrial insects excrete waste protein nitrogen mainly in the form of uric acid. Among aquatic insects, however, a reversal to ammonia as the main end-product of protein catabolism appears to be widespread (Staddon, 1955), although some variation in nitrogen excretion is to be expected since the freshwater habitat has been colonized independently at the ordinal, and in some cases family, level. In some species it would not be surprising to find that waste protein nitrogen (α-amino-N) is in part excreted as ammonia and in part transformed into uric acid before expulsion. It was the primary object of the present work to clarify the position in nymphs of *Aeshna*.

This paper also includes some preliminary observations on nitrogen gain after feeding.

MATERIAL

Nymphs of *A. cyanea* (Müll.) were collected from a small pond in Northumberland, near Newcastle upon Tyne. A few adults were bred from nymphs to ensure proper identification. I am greatly indebted to Dr E. T. Burtt of the Zoology Department, King's College, Newcastle upon Tyne, for kindly arranging the collection and dispatch of living material when required.

Animals were starved in clean water at 20° C. for a period of 7–14 days before experiment. During this period unabsorbed food materials were eliminated from the gut.

METHODS

(*a*) *Collection of excreta*. The method consisted in partly immersing a nymph in 2·5 ml. of an acetate buffer solution at pH 3·7 for a period of 24 hr. at 20° C. The stock solution contained 13·6 g. sodium acetate $3H_2O$ and 44 ml. acetic acid/100 ml. This solution was diluted 1/150 with distilled water before use.

The choice of an acetate buffer solution was determined by the need to prevent decomposition of the excreta during the period of collection. Using distilled water it is estimated that as much as 20 and 80%, respectively, of the total quantities of ammonia and uric acid excreted may be lost during a 24 hr. collecting period.

Decomposition of the excreta in distilled water is attributed to the presence of contaminating micro-organisms derived from the gut or body surface of the animal, although a small quantity of ammonia may be lost by diffusion into the air. The low pH of the acetate buffer solution both prevents loss of ammonia by diffusion and makes conditions unfavourable for micro-organisms; acetic acid has a disinfectant action independent of pH, a property of the whole molecule or anion (Wilson & Miles, 1946).

The efficacy of the acetate buffer solution was tested. Samples containing excreta, collected during a period of 24 hr., were cultured using a routine bacteriological technique. The samples appeared to be sterile (in contrast, washings taken from nymphs with sterile distilled water yielded heavy growths of bacteria). There was no detectable loss of ammonia in samples left for a further period of 24 hr. at 20° C. after the nymphs had been removed. Uric acid recoveries of over 90% were obtained in tests in which uric acid was added to the medium before insertion of the nymphs. Since the acetate buffer solution preserves the excreta and in no way appears to affect the vitality of nymphs which were at times immersed continuously for over a week, it was consequently considered entirely satisfactory for the purpose of the present investigation.

The elimination of urine from the gut appears to occur frequently. Measurements of the ammonia content of the medium, made at 90 min. intervals, rarely failed to reveal an increase in ammonia level. This emphasizes that daily measurements must approximate closely to the actual daily output of excreta, negligible error being incurred through retention in the gut.

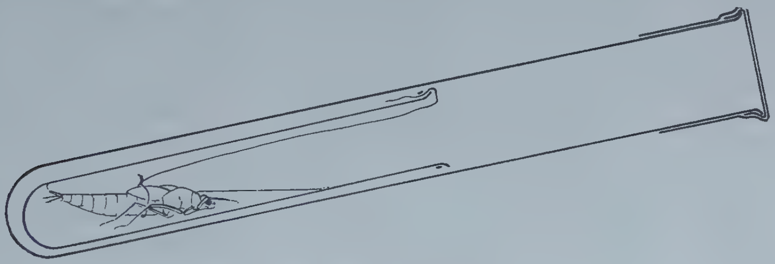

Fig. 1. Apparatus for collecting excreta.

The apparatus employed for the purpose of collecting excreta is shown in Fig. 1. The inner tube, of internal dimensions 11·4 × 1·4 cm., is made of 'Pyrex' and bears a 5·0 ml. graduation mark. In this tube the excreta are collected and later incinerated if a total nitrogen determination is required. After the introduction of 2·5 ml. of the buffer solution the nymph is secured in position with nichrome wire. One end of the wire is tied round the 'waist' of the animal, the other is folded over the mouth of the tube and gripped by an elastic band. The head of the nymph is directed towards the mouth of the tube. This prevents loss of fluid which may be voided forcibly from the rectum. The outer tube, approx. 20 × 2·5 cm., serves to keep the inner tube clean when immersed in a water-bath and also increases the volume of air available. The mouth of the outer tube is covered, first with a piece of sheet

Polythene, secured with an elastic band, and then with the cut end of a rubber balloon to make it completely water-tight. The apparatus is inclined at an angle of 5–10° from horizontal to make surface breathing possible.

At the end of the 24 hr. collecting period the inner tube is removed and clamped in a vertical position. The nymph is drawn half-way up the tube and then washed with water or with a solution of sodium carbonate anticipating uric acid estimations. The nymph is provoked to expel fluid retained in the rectum. The washing is then repeated. The nymph is now removed, the tube centrifuged to collect any fluid adhering to the sides, and the volume made up to 5·0 ml. with distilled water. Transfer of the nymph from one tube to another is effected with speed and without loss of fluid adhering to the body surface.

Faeces nitrogen was estimated separately. Since faeces are constrained within a peritrophic membrane they can be transferred intact to a clean tube by means of a hooked glass rod.

Parallel experiments were periodically carried out in which nymphs were absent.

(*b*) *Analytical methods*. Ammonia nitrogen was at first estimated by the diffusion/titration method of Shaw & Beadle (1949), and later by the diffusion/conductimetric method of Shaw & Staddon (1958). A comparison of results obtained by the diffusion/titration method with results obtained by the Nessler colorimetric method showed that ammonia was the only volatile base separated in the diffusion process.

Total nitrogen was estimated by a micro-Kjeldahl method. After removal of samples for ammonia analysis the fluid remaining was acidified, evaporated overnight, digested vigorously for a period of 1 hr. and then made up to 5·0 ml. with distilled water. The ammonia content was determined by one of the methods noted previously. For the ratio potassium sulphate/sulphuric acid in the digest mixture see McKenzie & Wallace (1954). Copper sulphate was used as catalyst. A mean recovery of 99·3% (min. 98; max. 101) was obtained in three recovery tests performed on a standard solution of ammonium sulphate. A mean recovery of 98·8% (min. 97; max. 99·4) was obtained in four tests on tryptophane. In estimating the total nitrogen content of the whole sample no correction was considered necessary for the very small quantity of fluid removed for ammonia analysis.

Uric acid was estimated by the method of Brown (1945) as modified by Dresel & Moyle (1950). Estimations were made in duplicate using 2 ml. samples. The procedure of Dresel & Moyle (1950) was used for extracting uric acid from whole nymphs. The method was tested in the manner described by these authors and was similarly found very satisfactory.

(*c*) *Method of feeding*. Nymphs were quantitatively fed on heat-coagulated egg-white. The dry weight of egg-white is about 12% of the wet weight and the bulk of the dry material is in the form of protein. A 100 mg. (wet weight) contains approximately 1·7 mg. nitrogen. Samples were obtained from the middle thick white (Brooks & Taylor, 1955) and weighed to the nearest 0·5 mg. on a 500 mg. torsion balance. The Kjeldahl method was used to determine the nitrogen content.

Feeding was accomplished by hand under the low power of a binocular micro-

scope. The nymph is held in one hand, ventral side uppermost. With forceps held in the other hand a portion of the weighed sample of egg-white is inserted between the distal extremities of the labrum and labium. By stroking the edge of the labrum the animal is induced to feed. The egg-white is seized by the maxillae from whence it is transferred to the mandibles, briefly masticated then swallowed. Further pieces of egg-white are given to the animal until the whole sample has been ingested, care being taken to see that no small particles remain trapped behind the mouth under the labium.

(*d*) *Collection of mid-gut contents*. Unabsorbed food in the midgut is collected in the following way. The animal is killed by crushing the head and then dissected to expose the gut. The body cavity is washed with saline. Surplus fluid is then removed from the surface of the gut with filter-paper. The contents are exposed by making a small incision in the midgut wall, care being necessary to prevent damage to the peritrophic membrane. Gut contractions cause the contained mass to protrude through the incision. The midgut contents are now removed entire within the peritrophic membrane by gently seizing and pulling the protruding portion with blunt forceps.

RESULTS

(*a*) *The excretion of ammonia*

Daily measurements were made to determine the ammonia nitrogen and total nitrogen output of nymphs for a period of 2 days during fasting and then for a further period of 3–5 days after feeding on egg-white (Table 1; Fig. 2).

The total nitrogen output prior to feeding ranged from 2·6 to 16·6 μg. (av. 7 μg. N)/100 mg. wet weight/24 hr. at 20° C. Of the total nitrogen excreted 41–91% (av. 74%) was in the form of ammonia.

Feeding was followed by a large increase in nitrogen output which lasted for 1 or 2 days. The total nitrogen output measured at the end of the first day after feeding ranged from 17·7 to 46 μg. (av. 32 μg. N)/100 mg. wet weight. Of the total nitrogen excreted 78–96% (av. 87%) was in the form of ammonia.

The non-ammonia nitrogen output prior to feeding ranged from 0·6 to 5·2 μg. (av. 1·8 μg. N)/100 mg. wet weight/24 hr. Measurements obtained at the end of the first day after feeding ranged from 0·7 to 9·8 (av. 3·9 μg. N)/100 mg. wet weight. This increase is small when compared with the increased output of ammonia after feeding.

The results clearly show that ammonia is quantitatively the most important nitrogenous excretory substance in the excreta of nymphs, both during starvation and after feeding on egg-white.

(*b*) *The excretion of uric acid*

It is evident from results shown in Tables 2 and 3 that uric acid is quantitatively a minor excretory product in nymphs of *A. cyanea*. The uric acid output of fasting nymphs ranged from 6·2 to 13·1% (av. 8%) of the total ammonia nitrogen and uric acid nitrogen output, and no increase in output was apparent after feeding on

Table 1. *The excretion of nitrogen before and after feeding on egg-white*

Exp. no.	Weight of nymph (mg.)	N ingested (μg.)	N in midgut at end exp. (μg.)	Excretory and faeces N (μg.)	Days before feeding		Days after feeding						
					1	2	1	2	3	4	5	6	7
1*	860	Approx.	—	Total N (excluding faeces)	32	22	152	123	36	36	—	—	—
			—	Ammonia N	20	9	146	100	24	27	—	—	—
				Faeces N	—	—	—	—	—	—	—	—	—
2	531	372	—	Total N (excluding faeces)	67	54	218	62	49	39	—	—	—
				Ammonia N	59	41	197	47	38	26	—	—	—
				Faeces N	—	—	—	—	—	5	—	—	—
3	510	263	—	Total N (excluding faeces)	16	21	141	27	17	—	—	—	—
				Ammonia N	12	16	136	21	10	—	—	—	—
				Faeces N	—	—	—	2	—	—	—	—	—
4	506	392	—	Total N (excluding faeces)	62	51	204	59	40	33	—	—	—
				Ammonia N	52	41	166	37	31	25	—	—	—
				Faeces N	—	—	—	—	—	—	—	—	5·5
5	326	273	—	Total N (excluding faeces)	54	36	149	67	35	—	—	—	—
				Ammonia N	37	19	117	45	28	—	—	—	—
				Faeces N	—	—	—	—	—	—	—	2	—
6	—	—	Appeared empty	Total N (excluding faeces)	32	43	147	84	73	78	14	Moult	
				Ammonia N	22	39	140	75	50	70	42		
				Faeces N	—	—	—	—	14	—	—		
7	830	1138	230	Total N (excluding faeces)	52	45	340	155	65	88	—	—	—
				Ammonia N	41	27	285	135	52	70	—	—	—
				Faeces N	—	—	—	14	—	7	—	—	—

* Animal 1 kept in water. Total N estimations in quadruplicate on small samples using method of Shaw & Beadle (1949).

egg-white (Table 2). The uric acid content of whole nymphs (Table 3) ranged from 5·3 to 9·3 μg. N/100 mg. wet weight. These quantities are approximately equivalent to a 24 hr. output of ammonia nitrogen during starvation.

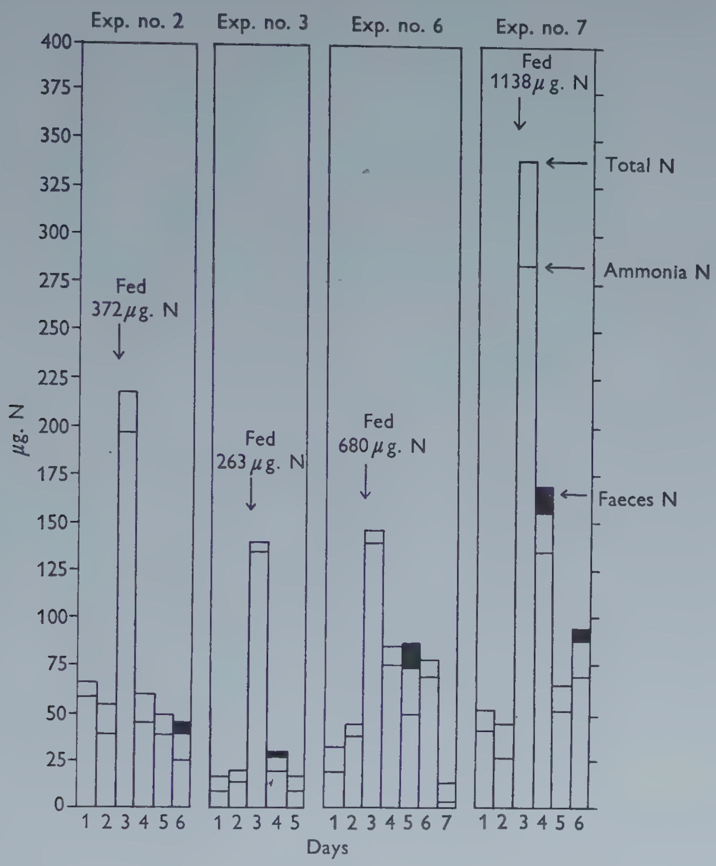

Fig. 2. Total N and ammonia N excretion during starvation and after feeding on egg-white.

It is of interest here to note that Florkin & Duchâteau (1943) failed to find uricase in extracts of *Aeshna* nymphs thereby indicating that uric acid is not degraded before excretion. The results obtained in the present work are taken to confirm this.

(*c*) *Nitrogen gain after feeding*

Estimates have been obtained of the nitrogen gained by nymphs after feeding on egg-white.

That the nitrogen ingested is ultimately wholly absorbed is indicated by the fact that faeces expelled after feeding contain very little nitrogen (Table 1). It became apparent, however, that a portion of the egg-white ingested may remain unabsorbed in the midgut for some days after feeding, and that the elimination of faeces (and fall in nitrogen output after the post-feeding peak) may not necessarily

Table 2. *The excretion of uric acid during starvation and after feeding on egg-white*

Exp. no.	Weight of nymph (mg.)	N excreted (μg. 24 hr.)	Starved	Fed	Starved	Fed
1	830	Ammonia N	41	200	58	123
		Uric acid N	3·6	3	5	2·7
		$\frac{\text{Uric acid N} \times 100}{\text{Ammonia N} + \text{uric acid N}}$	8·1	1·5	8	2·1
2	855	Ammonia N	29	52	5·3	76
		Uric acid N	2·5	2·1	0·8	2·8
		$\frac{\text{Uric acid N} \times 100}{\text{Ammonia N} + \text{uric acid N}}$	7·9	3·9	13·1	3·5
3	875	Ammonia N	9·2	170	38	182
		Uric acid N	1·3	2·8	2·5	5·5
		$\frac{\text{Uric acid N} \times 100}{\text{Ammonia N} + \text{uric acid N}}$	12·4	1·6	6·2	2·9

Table 3. *The uric acid content of whole nymphs*

Exp. no.	Days starved	Weight (mg.)	No. of animals	Mean weight and weight range (mg.)	Uric acid content (μg. N/100 mg. wet weight)
1	12	2090	8	261 (195–292)	7·2
2	12	1340	10	134 (111–168)	8·7
3	12	1310	12	109 (85–121)	9·3
4	15	2265	4	553 (440–735)	5·4
5	15	2465	4	616 (455–715)	5·3
Av.					7·2

be associated with complete absorption of the egg-white ingested. Consequently, the estimation of nitrogen gain over a short period necessitates an estimation of the nitrogen remaining unabsorbed in the gut at the end of the selected experimental period.

In Exp. 6 (Table 1) the nymph moulted on the sixth day after feeding and died, presumably due to asphyxiation since withdrawal of the abdomen from the old cuticle was precluded by the presence of the securing wire. The ingested protein appeared to have been completely absorbed since the gut was empty but for the presence in the midgut of a number of peritrophic sacs concentrically disposed one within the other. It can be calculated, therefore, that during the 6 days after feeding the nymph gained 270 μg. nitrogen, an amount equivalent to 40% of the nitrogen ingested and absorbed.

In Exp. 7 (Table 1) the nymph was killed and dissected 4 days after feeding. The foregut appeared empty, but the midgut was partially distended with semi-

liquid, homogeneous, pale yellow-brown contents, presumed to be partially digested egg-white (it is to be noted that faeces had already been expelled on the second and fourth days after feeding). The mass within the midgut was removed entire within the peritrophic membrane and estimated to contain 230 μg. nitrogen. From the figures included in Table 1 it can be calculated that, of the nitrogen ingested, 22% remained unabsorbed 4 days after feeding. Further, it can be calculated that the quantity of nitrogen gained during the 4-day period after feeding amounted to 239 μg., an amount equivalent to 26% of the total nitrogen absorbed.

Table 4 includes further data on nitrogen absorption, nitrogen gain and nitrogen excretion after feeding. The foregut in all cases appeared empty at the end of the experimental period but unabsorbed protein still remained in the midgut. In one case there was a small nitrogen deficit, in the remaining three cases the quantity of nitrogen gained ranged from 10 to 23% of the total nitrogen absorbed.

Table 4. *The excretion of nitrogen and nitrogen gain after feeding*

Exp. no. ...	1	2	3	4
Weight of nymph (mg.)	530	533	984	1015
Time (hr.)	48	48	22	42
N ingested (μg.)	230	287	301	301
N of mid-gut contents (μg.)	63	84	99	124
N absorbed—apparent value, obtained by difference (μg.)	167	203	202	177
N absorbed (% N ingested)	73	71	67	59
Total N excreted (μg.)	139	156	181	182
N gain (μg.)	28	47	21	−5
N gain (% apparent N absorbed)	17	23	10	—
Ammonia N excreted (% total N)	83	79	73	71

DISCUSSION

The results presented in this paper affirm that ammonia is the main end-product of protein catabolism in nymphs of *A. cyanea*. Uric acid, a minor excretory product, is taken to originate solely from the breakdown of purines. The alternative possibility, that uric acid is in part derived synthetically from the α-amino-N of protein, is discounted on the grounds that output does not increase after feeding on a protein-rich diet. It is understood that measurements on the excreta give a true indication of output since measurements on extracts of whole nymphs revealed that very little retention is occurring.

Feeding is followed by a large, temporary increase in the amount of ammonia excreted. It has been estimated that during the 24–48 hr. period after feeding a quantity of nitrogen is excreted equivalent in amount to the greater part of the food nitrogen absorbed during that period. It would be of interest to take this aspect further since little is known about the factors influencing nitrogen excretion and retention in insects. For the purpose of investigating these problems *Aeshna* nymphs would appear to offer admirable material.

SUMMARY

1. The excretion of ammonia and uric acid has been studied in nymphs of *Aeshna cyanea* (Odonata, Anisoptera).

2. Ammonia is the main nitrogenous component of the excreta of nymphs during fasting and after feeding on a protein-rich diet. Only a small proportion of the total nitrogen excreted is present as uric acid.

3. Retention of uric acid in the body is at most trivial.

4. When fasting nymphs are fed on a protein-rich diet in the form of egg-white there is a large, temporary increase in the amount of ammonia excreted, but the output of uric acid remains constant.

5. It has been estimated that nymphs excrete a quantity of nitrogen within 24–48 hr. after feeding equivalent in amount to 60% or more of the total nitrogen absorbed during that period.

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PIGMENTATION OF AN OSTRACOD, *HETEROCYPRIS INCONGRUENS*

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(*Received* 30 *April* 1959)

INTRODUCTION

The information available concerning the pigments of ostracods is meagre. Fox (1948) records the presence of haemoglobin in the blood of *Cypria ophthalmica* (Jurine) and later (1957) in the blood of *Pseudocypris*. Fox (1955) also records the presence, in *Cypris pubera* (O. F. Müller), of a green pigment showing a reversible colour change to brown in alkaline conditions, and a similar change when reduced with sodium dithionite. The chemical nature of this pigment is unknown.

There are numerous references to the colours of ostracods in the systematic literature, and some particularly fine coloured plates in the works of G. O. Sars, but there is no indication of the chemical nature of the pigments involved. In one of Sar's papers (1894) there is a coloured figure of a *Heterocypris* species (plate IV, under the name *Cypris sydneia* King) which gives a very good impression of the living animal. In a later paper (1924) Sars remarks that the genus *Heterocypris* is notable for its yellow colour; this being a feature by which it may be distinguished from most other ostracods.

Because of this remarkable coloration, and because the species sometimes occurs in numbers large enough to permit the application of chemical methods, it was decided to investigate the pigments of *H. incongruens* (Ramdohr).

MATERIAL AND METHODS

The stock of ostracods came from a large aquarium tank at Bedford College. This tank contained mud from an unknown locality, and the only other animals in the tank were a few small copepods and rhabdocoels. In the spring of 1958 *H. incongruens* became very numerous, and samples were taken in order to extract pigments. The ostracods remaining in the tank were fed with various algae which grew in other aquarium tanks containing axolotls and catfish.

When controlled feeding was desired the unicellular green alga *Chlorella vulgaris* Beij was used. This alga was grown in pure culture on agar slopes in front of a mercury vapour lamp. In certain other experiments the filamentous blue-green alga *Anabaena cylindrica* Lemm. was used; this was also grown in pure culture on agar slopes.

A number of the ostracods were reared individually in standard conditions similar to those used in a previous study of the growth of *Daphnia* (Green, 1956).

The only difference was that the standard suspension of *Chlorella* was allowed to settle on the bottom of the culture tube and was not stirred as it was in the study of *Daphnia*.

When a sample of ostracods was taken the animals were passed through several changes of clean water over a period of about half an hour. This enabled a substantial amount of material to be eliminated from the guts of the ostracods so that the pigment analysis was not complicated by pigments from the plant material in the gut. Various methods of extracting pigments were tried; details are given in the relevant section. Absorption spectra were examined in a Unicam S.P. 500 spectrophotometer with an ultra-violet attachment.

LOCATION OF PIGMENTS

The general colour of *Heterocypris incongruens* is orange yellow, with a tendency towards brownness on the upper parts of the shell valves. The gut often appears as a brownish mass inside the body, while the gut diverticula, one in each shell valve, appear as dark streaks against the general yellow background. There is much orange yellow pigment in a granular form in the epidermal cells of the shell valves, and finer granules of a similar pigment in the locomotory limbs and furca. The blood is also orange yellow; here the pigment is in solution and not granular. The eggs, which can sometimes be seen in the ovary, vary in colour from pale orange to bright scarlet. When the eggs are scarlet the pigment can be seen to be located in two different types of droplets. The larger droplets are orange in colour and are about 2 or $4\,\mu$ in diameter, while the smaller droplets are only about $0{\cdot}5\,\mu$ in diameter and are bright scarlet in colour. It has not been possible to investigate the pigments of the eggs separately, but it is probable that they are carotenoids, because they give a blue colour with sulphuric acid and are soluble in acetone and petrol ether.

When *H. incongruens* is reared at a temperature of 22° C. and fed on *Chlorella* the ovary begins to appear orange or red after the animal has passed through seven moults and is about 16 days old. The first eggs are cast off attached to the shell valves at the next moult. After this the animal does not moult again and subsequent batches of eggs are carried within the shell valves for a variable period or else laid on some solid object near the surface of the water. The first eggs remain attached to the shell valves for about a week until the young ostracods emerge. The main difference from the adult pigmentation is that the young are paler, and the gut diverticula do not appear in the shell valves until the fifth instar (Schreiber, 1922).

LACK OF HAEMOGLOBIN

Examination with a microspectroscope has failed to detect haemoglobin in *Heterocypris incongruens*. This was so even when the ostracod was well fed and kept in water containing less than 1·0 ml./l. of oxygen for 2 weeks.

Attempts were also made to produce a pyridine haemochromogen, by the addition of sodium dithionite and pyridine. The microspectroscopic technique used was sensitive enough to demonstrate the presence of haem in a single egg of a pale

cladoceran such as *Diaphanosoma*, but it failed with over twenty specimens of *Heterocypris*. It is clear that haem pigments are absent, or very scarce, in this ostracod. This is in contrast with the species examined by Fox (1948, 1957) where haemoglobin is easily detectable in a single specimen.

The ability of *H. incongruens* to survive, and indeed flourish, at low oxygen concentrations has been demonstrated by Fox & Taylor (1955). One of my own experiments, originally designed for another purpose, has accidentally demonstrated that this ostracod can endure anaerobic conditions for a considerable period. The oxygen concentration in one of the experimental flasks fell so low that it became undetectable by the micro-Winkler technique of Fox & Wingfield (1938), and the water smelt strongly of hydrogen sulphide. In spite of this the ostracods flourished, and after 2 weeks one specimen was found with 32 eggs within its shell valves. Numerous eggs had been deposited on the wall of the flask just at the surface of the water. It is clear that *H. incongruens* can live and reproduce at extremely low oxygen concentrations without the aid of any detectable concentration of haem pigments. This point will be taken up again in the discussion.

CAROTENOIDS

Acetone extracts from several thousand ostracods yielded an orange solution, from which the pigment was taken into petrol ether after dilution of the acetone with water. After drying the petrol ether solution over anhydrous sodium sulphate, a phase test with 90% methanol showed the orange pigment to be entirely epiphasic. When chromatographed on an alumina column the pigment resolved into two bands, both of which passed through the column with petrol ether, but the second took much longer than the first. The absorption spectrum of the first fraction in hexane showed a shoulder at 426 mμ and distinct peaks at 450 and 477 mμ. This is in good agreement with the absorption spectrum of β-carotene given by Karrer & Jucker (1950). The second fraction showed a single absorption peak at 468 mμ in hexane. This suggests an astaxanthin ester. When the fraction was saponified with potassium hydroxide in methanol the pigment became hypophasic when tested against petrol ether, and remained hypophasic in the presence of excess water. When the hypophase was acidified with glacial acetic acid the pigment passed rapidly to the epiphase. This behaviour is characteristic of astacene liberated from an astaxanthin ester.

The diet of the ostracod included green and blue-green algae. The food would thus contain a variety of xanthophylls; for instance: *Chlorella vulgaris* has about 75% of its carotenoids in the form of lutein and violoxanthin, and a further 10% in the form of neoxanthin (Goodwin, 1954), *Anabaena cylindrica* has 17% of its carotenoids in the form of myxoxanthophyll (Goodwin, 1957). There was no indication of the presence of any of these hypophasic pigments in the extracts from the ostracods.

PTERIDINE

After repeated acetone extraction the residue was still yellow in colour; when it was treated with an ammonia solution a pale yellow pigment was extracted but faded rapidly and became colourless. The pale yellow solution and the colourless solution resulting from it had a brilliant blue fluorescence when exposed to ultraviolet light (365 mμ). This fluorescence was quenched by the addition of sodium dithionite, but returned when the solution was shaken with air. The fluorescence was destroyed by the addition of strong acid or alkali.

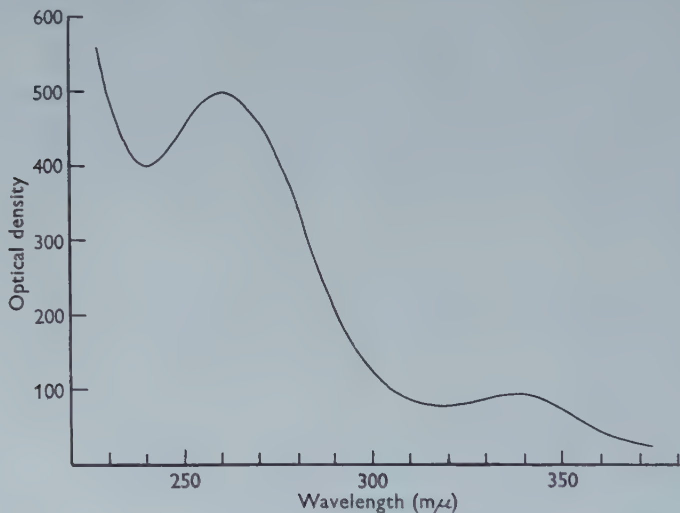

Fig. 1. Absorption spectrum of blue fluorescent ammonia extract from *Heterocypris incongruens*.

An equal volume of absolute ethanol (spectroscopically pure) was added to the ammonia extract and the mixture was filtered. The ultra-violet absorption spectrum of the filtrate was examined, using a mixture of ammonia and absolute ethanol as the blank. The result is shown in Fig. 1. There is a distinct absorption maximum at 260 mμ and a low rounded hump with maximum absorption at 338 mμ. This absorption curve is consistent with the presence of a pteridine (cf. Mason, 1954).

The colourless solution was chromatographed on paper using a 4:1:5 butanol–acetic acid–water mixture as the developing solvent. This usually resulted in a single fluorescent spot with an R_F value of 0·4, but on some occasions traces of a second spot were seen; this may have been an artifact due to trailing of the fluorescent substance in the developing mixture.

It was thought that the colourless fluorescent substance might be 2-amino-4-hydroxypteridine-6-carboxylic acid (pterincarbonic acid), which was obtained by Forrest & Mitchell (1954) from a photolabile yellow pigment in *Drosophila melanogaster*, and which Ziegler-Günder (1956) found as a derivative from a yellow pteridine in the skin of *Rana temporaria*. This possibility was tested by a series of

chromatograms, using ammonia extracts of frog skin which had been exposed to light before starting the chromatogram. In this way it was found that the ostracod pteridine migrated more quickly than the spot which Ziegler-Günder identified as pterincarbonic acid. Further, the fluorescence of the substance from the ostracod is somewhat more violet in colour. The precise identification of the fluorescent pigment of *Heterocypris* must await the availability of much larger amounts of material, but meanwhile the evidence presented above indicates that it is a pteridine.

The distribution of the fluorescent pigment in the body of the ostracod was examined by dissecting the animals in an ammonia solution under an ultra-violet lamp. It was found that the pigment is distributed throughout the body, even in the limbs and blood, but is absent from the eggs.

BILADIENE IN THE GUT WALL

The ostracods from the aquarium tank were found on dissection to have dark blue granules in the cells of the gut epithelium, both in the central portion of the gut and in the diverticula extending into the shell valves. The granules were more or less rounded, with a diameter varying from 0·5 to 3 μ. When treated with yellow concentrated nitric acid a brilliant Gmelin reaction resulted. The granules became a brighter blue, then successively purple, red, orange and yellow. This is a clear indication that a bile pigment is accumulated in the gut wall.

A large sample of the ostracod from the aquarium tank was ground up with a 19:1 methanol–sulphuric acid mixture, giving a bluish solution. The blue pigment was taken into chloroform after dilution of the methanol with water. The solution in chloroform had a red fluorescence in ultra-violet light, but unlike the red fluorescence of porphyrins this was quenched by the addition of acid. The addition of zinc acetate and a little iodine in methanol did not alter the fluorescence, but when more iodine was added the fluorescence became green. This behaviour of the pigment is consistent with that of a biladiene (Lemberg & Legge, 1950; Comfort, 1950). Unfortunately there was not enough of the pigment to measure its absorption spectrum.

A series of experiments was made to see what factors might influence the accumulation of the biladiene in the gut wall. Groups of ostracods were separated into dishes containing *Chlorella* as food. Some dishes were kept in the dark, some in front of a mercury vapour lamp, and some of the ostracods were kept in conical flasks in the dark to reduce the oxygen content of the water. The same clear result was obtained in each group. After 6 days the amount of biladiene in the gut wall had decreased to about half the original amount, and after 9 days the gut wall was colourless. The biladiene is evidently not derived from green algae, and continued feeding on such algae leads to the eventual disappearance of the bile pigment.

The next step was to investigate the mud of the aquarium tank to see if any blue-green algae were present; the search resulted in the finding of small amounts of a dark blue species of *Oscillatoria*. The ostracods were evidently accumulating the bile pigment from the phycobilin in this alga. Final proof of this was given when

specimens of the ostracod were reared from birth on a diet of *Chlorella*, and then transferred to a diet of the blue-green *Anabaena cylindrica*. The gut wall of the animals feeding on *Anabaena* became a bright blue green, while the gut wall of control specimens feeding on *Chlorella* remained colourless.

A significant point which emerged from this experiment was that the colour of the gut wall varied with the species of blue-green alga on which the ostracods were feeding. When the dark blue *Oscillatoria* was eaten the gut wall was dark blue, and when the lighter blue-green *Anabaena* was eaten the gut wall was correspondingly paler. It seems that the phycobilins of the blue-green algae are accumulated in the gut wall of the ostracod without much change in their constitution.

Experiments were also made to see if other species of ostracods could accumulate phycobilins. The ostracods were kept in dishes with a mixed diet of *Chlorella* and *Anabaena*. Samples of each species were examined at intervals to see if the gut wall was coloured; if a blue green colour was present its identity was confirmed with the Gmelin reaction. In this way it was found that the following species can accumulate phycobilins: *Cypridopsis vidua* (O. F. Müller), *Herpetocypris reptans* (Baird), *Cyclocypris ovum* (Jurine), and an unidentified species of *Candona*.

DISCUSSION

The absence of haemoglobin from this ostracod, coupled with its ability to survive in anaerobic conditions indicates that the intermediate pathways of oxygen transfer differ from the usual haemoglobin-cytochrome system. The form that the alternative pathway may take is unknown, but it may possibly involve the fluorescent pigment which shows a reversible reduction on the addition of sodium dithionite. This property of reversible oxidation and reduction might well play some part in the intermediate metabolism of the ostracod under anaerobic conditions.

The occurrence of pteridines in decapod crustaceans has been surveyed by Busnel & Drilhon (1948). They found fluorescent pigments associated particularly with the darker pigments, both melanins and ommatines. It is possible that the brownness which sometimes appears on the upper parts of the shell valves of *Heterocypris* is due to an ommatine. The method used to extract the blue fluorescent substance would also extract ommatines, and the ultra-violet absorption of such pigments would reinforce the peak found at 260 mμ.

There does not appear to be any information available in the literature concerning the presence of pteridines in the Entomostraca. My own preliminary investigations have failed to find any easily detectable amounts of such substances in the cladoceran, *Daphnia magna*, or in an anostracan, *Artemia salina*. This was in spite of using numbers of these species greatly in excess of the numbers of *Heterocypris* which gave the brilliant blue fluorescence.

There does not seem to be any previous literature on the carotenoids in ostracods. The occurrence of β-carotene and astaxanthin esters is rather what might be expected from the little knowledge we have of carotenoids in other Entomostraca. The cladoceran *Daphnia magna* contains both β-carotene and astaxanthin (Green,

1957), and both these pigments have been found in certain copepods (Goodwin & Srisukh, 1949; Batham, Fisher, Henry, Kon & Thompson, 1951).

Bile pigments are of infrequent occurrence in the Crustacea: biliverdin is the only one which has been identified. It is found in the liver of the North American crayfish *Cambarus* (Bradley, 1908), and in the roots of certain parasitic cirripedes (Raphael, 1948; Fox, 1953). It might be thought that various other small crustaceans would have the ability to accumulate phycobilins from blue-green algae in their food, but I have not noticed any such accumulation in any freshwater cladoceran or copepod collected over a period of several years. The small cladoceran *Chydorus sphaericus* has been reared through several generations on a pure diet of *Anabaena cylindrica*, which proved to be an excellent food, but no trace of phycobilin accumulation in the gut wall has been found.

SUMMARY

1. *Heterocypris incongruens* contains at least three different types of pigment: carotenoids, a pteridine, and a bilin. Haemoglobin and other haem pigments appear to be lacking in this species.

2. Astaxanthin and β-carotene are the only carotenoids found, even when the ostracod is feeding on algae containing abundant xanthophylls of various types.

3. A yellow pteridine, which rapidly decolorizes after extraction, is widespread in the body of the ostracod, but not in its eggs. It is suggested that this substance may play a part in the intermediate metabolism of the ostracod when in anaerobic conditions. *Heterocypris* can live and reproduce in anaerobic conditions for at least 2 weeks.

4. Biladiene pigments accumulate in the gut wall when the ostracod feeds on blue-green algae. These pigments can be made to disappear from the gut wall by restricting the diet to green algae, then made to reappear when a blue-green alga is given as food.

My thanks are due to Dr G. E. Fogg for his generous gift of a pure culture of *Anabaena cylindrica*, from which my own cultures were started. Prof. H. Munro Fox, F.R.S., and Dr Barbara M. Gilchrist have kindly read and criticized the manuscript.

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(ii) *Text half-tone blocks.* These are suitable for illustrations involving brushwork, or in which the depth of shading is an essential feature. They can be used for such things as oscillograph records and some photographs can be produced in this way, a good glossy bromide print being required. Illustrations should be gummed on *white* card, grouped and numbered as they are to appear in print. All lettering should be shown in position on a covering sheet of transparent paper.

(iii) *Plates.* Plates should be used only for illustrations, such as photomicrographs, in which the most accurate reproduction of fine detail is called for. Plates are expensive and the Editors may require an author to defray the cost of plates which in their opinion are not essential. The photographs making up the plate should be gummed on *white* card, grouped and numbered as they are to appear in print. Exclusive of margin the plate figures should not cover, when reduced, an area greater than 7½ in. in length × 5 in. in width when ready for reproduction as a single plate, or 7½ in. × 11½ in. in the case of double plates. All lettering should be shown in position on a covering sheet of transparent paper.

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PRINTED IN GREAT BRITAIN AT THE UNIVERSITY PRESS, CAMBRIDGE
(BROOKE CRUTCHLEY, UNIVERSITY PRINTER)